University of Alberta

NTPase and helicase activities of SCO3550 from *Streptomyces coelicolor*

by

Wisdom Kwaku Ayidzoe ©

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Abstract

sco3550 situated next to *bldG* in *Streptomyces coelicolor* encodes a putative RNA helicase that is predicted to be expressed from two identified, in-frame ATG and GTG translational start codons. The purified recombinant putative isoforms reflecting translation initiation at the ATG or GTG start codons, displayed similar biochemical activities. In an in vitro assay, the putative isoforms destabilized standard artificial dsRNAs in an energy-dependent manner. The RNA unwinding activity of the putative isoforms was supported by all eight common nucleoside triphosphates. Using a coupled spectrophotometric assay, the recombinant proteins were observed to display nucleic acid-dependent NTPase activity. These observations demonstrate that SCO3550 is a bona fide RNA helicase. This, together with the observed overlap between the *sco3550* promoter region and the promoter region of *bldG*, which encodes a developmental regulatory protein, suggests SCO3550 may be involved in remodeling of RNAs necessary for development in *Streptomyces coelicolor*.

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Table of Contents

CHAPTER 1:	**INTRODUCTION**	2
1.1. Overview of *Streptomyces*		2
1.2. Genes controlling morphological differentiation in *S. coelicolor*		4
1.3. RNA Helicases		8
1.4. Cellular functions of RNA helicases		13
1.4.1. Transcription		13
1.4.2. Translation		16
1.4.3. Ribosome biogenesis		17
1.4.4. Remodeling of ribonucleoproteins		19
1.4.5. RNA decay		20
1.4.6. mRNA splicing		22
1.4.7. Formation of small interfering RNAs		26
1.4.8. mRNA transport		27
1.4.9. Other functions		29
1.5. SCO3550, a putative RNA helicase from *S. coelicolor*		31
1.6. Project objective		32
CHAPTER 2:	**MATERIALS AND METHODS**	37
2.1. Bacterial strains, plasmids and growth conditions		37
2.1.1. *Escherichia coli* and *Streptomyces coelicolor* strains used		37
2.1.2. Plasmids and vectors used		37
2.1.3. Growth and maintenance of *Escherischia coli* strains		37

2.1.4. Preparation *of E. coli* glycerol stocks 37

2.1.5. Growth and maintenance of *S. coelicolor* strains 37

2.1.6. Preparation of *S. coelicolor* glycerol stocks 41

2.2. DNA isolation and transformation 41

2.2.1. Preparation of *E. coli* chemically competent cells 41

2.2.2. Transformation of *E. coli* 41

2.2.3. Isolation of plasmid DNA from *E. coli* 42

2.3. DNA purification and analysis 42

2.3.1. Restriction digestion and cloning of DNA 42

2.3.2. Polymerase chain reaction (PCR) 42

2.3.3. Agarose gel electrophoresis of DNA 43

2.3.4. Purification of DNA from agarose gels 43

2.3.5. Polyacrylamide gel electrophoresis of DNA 43

2.3.6. DNA sequencing 45

2.4. Protein isolation, purification and analysis 45

2.4.1. Over expression and purification of recombinant SCO3550 45

2.4.2. Preparation of *S. coelicolor* and *E. coli* cell extracts 46

2.4.3. Quantification of protein 46

2.4.4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) 46

2.5. Enzyme assays 48

2.5.1. Nucleotide triphosphatase (NTPase) assay 48

2.5.2. RNA helicase assay 48

2.5.3. in vitro RNA transcription assays 49

2.5.4. Preparation of RNA helicase substrates 50

CHAPTER 3: RESULTS 53

3.1. Expression and purification of recombinant SCO3550 53

3.2. RNA unwinding activity of SCO3550 62

3.3. Hydrolysis of NTPs by SCO3550 90

CHAPTER 4: DISCUSSION 98

CHAPTER 5: REFERENCES 110

CHAPTER 6: APPENDIX 130

6.1. Calculation of the concentration of MBP-ATGSCO3550 133

6.2. Calculation of the moles of the dsRNA substrate (Fig. 3.6A) used 133

6.3. Molar proportion of MBP-ATGSCO3550 to the RNA substrate 134

List of Tables

Page

Chapter 2

2.1. Bacterial strains used in this study 38
2.2. *E. coli* and *Streptomyces* plasmids used in this study 39
2.3. Oligonucleotide primers used in this study 44

Chapter 6

6.1. RNA-dependent NTPase activities of recombinant ATG-SCO3550 (Experiment 1) 130
6.2. NTP requirements for MBP-ATGSCO3550 RNA unwinding activity 132

List of Figures

Page

Chapter 1

1.1. Comparison of conserved motifs of the subfamilies of helicase superfamily II 11

1.2. Putative translational start codons of *sco3550*..... 34

Chapter 3

3.1. Strategy for creation of a His_6-ATGSCO3550 overexpression vector 55

3.2. Expression of His_6-ATGSCO3550 in *E. coli* 58

3.3. Purification of recombinant His_6-ATGSCO3550 61

3.4. Strategy used in the construction of pAU339 64

3.5. Affinity purification of recombinant MBP-SCO3550 66

3.6. Structure of dsRNA substrates used for the RNA helicase assays 69

3.7. His_6-ATGSCO3550 purified from *S. coelicolor* does not unwind dsRNA 71

3.8. Influence of enzyme concentration on 5' to 3' unwinding by SCO3550 73

3.9. Effect of incubation time on the 5' to 3' unwinding activity of SCO3550 75

3.10. Effect of ATP concentration on the 5' to 3' unwinding activity of SCO3550.... 78

3.11. NTP requirements for MBP-SCO3550 RNA unwinding activity..... 80

3.12. GST-BldG does not enhance the unwinding activity of SCO3550..... 83

3.13. Divalent cation requirements for MBP-SCO3550 RNA unwinding..... 85

3.14. Influence of enzyme concentration on 3' to 5' unwinding activity of SCO3550..... 88

3.15. Influence of nucleic acids on the ATPase activities of MBP-SCO3550..... 92

3.16. NTP hydrolysis by MBP-SCO3550..... 95

List of Abbreviations

α	Alpha
Δ	Delta or deletion
ε	Epsilon
κ	Kappa
λ	Lambda bacteriophage
μ	Micro
ω	Omega
ϕ	Phi
σ	Sigma factor
A	Adenine (base), Alanine (amino acid)
AMP-PNP	Adenosine 5'-(β,γ-imido)triphosphate
ATP	Adenosine 5'-triphosphate
ATPase	Adenosine triphosphatase
bp	Base pair(s)
BSA	Bovine serum albumin
C	Cytosine (base), Cysteine (amino acid)
C-	Carboxy-
°C	Degrees Celcius
cpm	Counts per minute
CTP	Cytidine 5'-triphosphate
D	Aspartic acid
Da	Dalton(s)
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
E	Glutamic acid
EDTA	Ethylenediamenetetraacetic acid
F	Phenylalanine
g	Gram(s)
G	Guanine (base), Glycine (amino acid)
GST	Glutathione *S*-transferase
GTP	Guanosine 5'-triphosphate

H, His	Histidine
HCV	Hepatitis C virus
HEPES	Hydroxyethylpiperazinethansulfonic acid
I	Inosine (base), Isoleucine (amino acid)
IPTG	Isopropyl β-D-thiogalactopyranoside
K	Lysine
kb	Kilobase(s)
kDa	Kilodalton(s)
L	Litre(s)
LB	Luria-Bertani medium
M	Molar, Methionine
MBP	Maltose-binding protein
MBSU	Molecular Biology Service Unit
mg	Milligram(s)
mL	Millilitre(s)
mM	MilliMolar
mQH_2O	MilliQ water
mRNA	Messenger RNA
NaOAc	Sodium acetate
NADH	Nicotinamide adenine dinucleotide, reduced form
NAD^+	Nicotinamide adenine dinucleotide, oxidized form
NDP	Nucleoside diphosphate
Ni-NTA	Nickel-Nitrilo-tri-acetic acid
nM	NanoMolar
nt	Nucleotide(s)
NTP	Nucleoside triphosphate
NTPase	Nucleoside triphosphatase
OD	Optical density
ORF	Open reading frame
P	Proline
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
poly A	Polyadenylic acid
poly C	Polycytidylic acid
poly U	Polyuridylic acid
poly dA	Polydeoxyadenylic acid
poly dC	Polydeoxycytidylic acid

poly dI	polydeoxyinosinic acid
poly dT	Polythymidylic acid
poly rI.polyrC	Polyinosinic-cytidylic acid
Q	Glutamine
R	Arginine
R2YE	Sucrose yeast extract medium
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
S	Serine
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
ss	Single-stranded
T	Thymine (base), threonine (amino acid),
TBE	Tris-borate-EDTA
TE	Tris-EDTA
tRNA	Transfer RNA
Trx	Thioredoxin (tag)
TTP	Thymidine 5'-triphosphate
U	Uridine
UTP	Uridine 5'-triphosphate
UV	Ultraviolet
V	Volts, Valine
Y	Tyrosine

CHAPTER 1:
INTRODUCTION

1.0. INTRODUCTION

1.1. Overview of *Streptomyces*

Streptomycetes are Gram-positive, soil dwelling, filamentous bacteria. They produce extracellular enzymes that degrade biopolymers in the soil making them the key organisms in the recycling of carbon (Bentley et al. 2002; Bertram et al. 2004). Besides their contribution to recycling of soil nutrients, streptomycetes produce several commercially important secondary metabolites. These secondary metabolites include herbicides, enzymes, and compounds with medical applications such as chemotherapeutic agents, immune suppressants, and antibiotics (Sheeler et al. 2005).

Among the members of the genus *Streptomyces*, *Streptomyces coelicolor* is the most studied and genetically well characterized (Kwakman and Postma 1994). *S. coelicolor* produces four well characterized antibiotics: a blue-pigmented polyketide actinorhordin, a red-pigmented tripyrole undecylprodigiosin, a lipopeptide calcium-dependent antibiotic and a cyclopentanone methylenomycin antibiotic (Sevcikova and Kormanec 2004). The production of two colored antibiotics makes *S. coelicolor* the choice for genetic manipulation of streptomycetes since the antibiotics serve as indicators for the onset of secondary metabolism (Sheeler et al. 2005). Interestingly, the genome of *S. coelicolor* shares much similarity with those of two pathogenic actinomycetes, *Mycobacterium tuberculosis* and *Corynebacterium diphtheriae* (Bentley et al. 2002). Common to these organisms are some putative developmental regulatory genes including genes that code for Wbl (WhiB-like) proteins that are present throughout the actinomycetes but absent from all other organisms so far sequenced (Molle et al. 2000; Soliveri et al. 2000). It is known that overexpression of a member of the wbl proteins

increases multi-drug resistance in *S. coelicolor* and *M. tuberculosis* (Morris et al 2003; Jakimowicz et al. 2004). The study of the regulatory genes in non-pathogenic, genetically manipulatable and harmless *S. coelicolor* would therefore provide an insight into fighting *Mycobacterium tuberculosis, Corynebacterium diphtheriae* and other related pathogens.

S. coelicolor, like other *Streptomyces spp*, undergoes a complex developmental life cycle that begins with the germination of their unigenomic spores, which subsequently develop into multigenomic substrate hyphae. Upon sensing an environmental signal, probably nutrient exhaustion, the substrate hyphae grow into erect multigenomic aerial hyphae (Willey et al. 1991). The later part of the life cycle is characterized by the differentiation of the tips of the aerial hyphae into grey-pigmented, UV resistant unigenomic spores. Formation of spores ensures survival and dispersal of the organism (Ryding et al. 1998). Two major sets of genes are implicated in the regulation of the life cycle: the *bld* (bald) genes that control aerial hyphae formation, and the *whi* genes that are required later for spore formation. Interestingly, morphological and physiological differentiation occur simultaneously in the course of development, suggesting common regulatory elements in controlling aerial hyphae formation and the production of secondary metabolites (Ma and Kendall 1994). In support of this observation, *bld* genes of *S. coelicolor* were identified by their mutant phenotype in which mutants failed to produce antibiotics and aerial hyphae (i.e. grew as unpigmented colonies with a smooth surface) (Merrick 1976).

1.2. Genes controlling morphological differentiation in *S. coelicolor*

whi genes are involved in the formation of mature spores in *S. coelicolor*. *whi* mutants fail to produce grey-pigmented spores at the tips of the aerial hyphae giving their colonies a whitish appearance, hence the name '*whi* genes' (Hopwood et al. 1970). *whi* genes are divided into two functional groups, namely early *whi* genes and late *whi* genes. The early *whi* genes comprise *whiA, whiB, whiG, whiH, whiI,* and *whiJ* and they regulate the beginning stages of the spore formation. The late *whi* genes include *sigF*, *whiD*, and *whiE* and they are required for the formation of the grey pigment and the maturation of the spores.

Homologues of *whiA* exist in all Gram-positive bacteria whose genomic sequences are known (Ainsa et al. 2000). The function of WhiA is however not yet known. *whiA* mutants usually have long and tightly coiled aerial hyphae and form few sporulation septa (Chater, 1972; McVittie, 1974). *whiB* encodes a putative transcription factor whose homologues exist in virtually all actinomycetes (Davis and Chater, 1992; Soliveri et al. 2000). Mutation in *whiB* produces a comparable phenotype as mutation in *whiA* (Chater, 1972; McVittie, 1974). The gene product of *whiG* is a sigma factor that is similar to the motility sigma factor of *Bacillus subtilis* (Chater et al. 1989). *whiG* mutants produce uncurved aerial hyphae that lack septa (Chater, 1972; McVittie, 1974). WhiH and WhiI negatively control their own synthesis; however, the former is a putative DNA binding protein while the latter is a response regulator-like protein (Ryding et al. 1998; Ainsa et al. 1999). *whiJ* encodes a small protein that is found in all streptomycetes (Gehring et al. 2000).

The expression of most of the late *whi* genes is dependent on the early *whi* genes. WhiD is a homologue of WhiB. Disruption of *whiD* culminates in the formation of few spores that are highly heat sensitive and irregular in size (Molle et al. 2000). The *whiE* locus is represented by eight genes, seven of which are cotranscribed and the eighth divergently transcribed from the operon. Mutants of the divergently transcribed gene of the *whiE* locus produce spores that are greenish in appearance (Davis and Chater, 1990; Yu and Hopwood, 1995). The last of the known late *whi* genes, *sigF,* expresses a late sporulation sigma factor and the mutants produce thin-walled, unpigmented spores that contain uncondensed DNA (Potuckova et al. 1995; Kelemen et al. 1996). Ryding et al. (1999) identified new sporulation loci, *whiK-whiO,* most of which are yet to be characterized. The disruption of *whiK* and *whiN* genes produced a bald phenotype. The *whiK* and *whiN* genes were therefore renamed as *bldM* and *bldN*, respectively (Molle and Buttner 2000; Bibb et al., 2000).

The formation of aerial hyphae in *S. coelicolor* is a result of an interplay of a large number of genes that express a diverse set of proteins (Talbot 2003). For example, the *chp* genes (for *coelicolor* hydrophobic aerial protein), *ram* genes (for rapid aerial mycelium), and the *bld* genes are all involved in aerial hyphae formation. The *chp* and *ram* genes encode hydrophobic structural molecules that aid the growth of aerial hyphae into the air while most of the *bld* genes encode regulatory proteins (Elliot and Talbot 2004; Hunt et al. 2005). The *chp* genes encode a family of 8 proteins, the chaplins, which share a hydrophobic domain (chaplin domain) of about 40 residues and are designated ChpA-H. Mutations in *chp* genes delayed aerial hyphae formation suggesting a role of the chaplins in the formation of aerial hyphae (Elliot et al. 2003). Chaplins form

a hydrophobic layer on the surface of the aerial hyphae and also act as surfactants allowing the aerial hyphae to break the surface tension at the air/water interface and extend into the air (Claessen et al. 2003; Claessen et al. 2004).

The *ram* genes were identified by Ma and Kendall (1994) when they introduced a DNA fragment carrying the *ram* genes from *S. coelicolor* into the wild type *S. lividans* that led to the induction of rapid aerial mycelium formation. The *ram* cluster is made up of five genes, four of which are located in an operon (*ramCSAB*), and a fifth gene, *ramR*, which is divergently transcribed from the operon (Keijser et al. 2002; O'Connor et al. 2002). The gene product of *ramR* activates the transcription of the *ramCSAB* operon (Keijser et al. 2002). *ramS* (Kodani et al. 2004) codes for a 42 amino acid protein that is post-translationally modified to form a protein previously named SapB (for spore associated protein B), *ramC* encodes a protein which probably functions as the SapB synthetase whilst the remaining two genes, *ramA* and *ramB*, express ABC transporter components that may be responsible for the transport of SapB (Kodani et al. 2004). Mature SapB is a 21 amino acid morphogenic peptide that acts as a biological surfactant like the chaplins. It reduces the water surface tension of the aqueous environment from 72 to 32mJ/m^2 thereby allowing the growth of aerial hyphae into the air (Waki et al. 1997; Kelemen and Buttner 1998; Tillotson et al. 1998; Wosten and Willey 2000; Elliot and Talbot 2004). Willey et al. (1991) observed that when purified SapB is added to the majority of the *bld* mutants or when the mutants are grown close to bacteria producing SapB, they regain formation of aerial mycelium. Furthermore, the growth of some of the *bld* mutants close to certain other *bld* mutants, leads to the production of SapB (Willey et

al. 1993). These observations suggest the involvement of *bld* genes in the production of SapB.

bld genes include *bldA* that encodes a leucyl tRNA that recognizes the UUA codon, a rare codon in *Streptomyces* (Lawlor et al. 1987). *bldB, bldC, bldD* and *bldM* encode well-characterized or putative DNA binding proteins (Hunt et al. 2005; Elliot et al. 1998; Elliot and Leskiw 1999; Molle and Buttner 2000) while *bldN* expresses an extracytoplasmic function sigma factor (Bibb et al. 2000). The gene product of *bldK* is likely to be an oligopeptide importer (Nodwell et al. 1996), and *bldG* encodes a 113 amino acid protein, which is a putative anti-anti-sigma factor. Divergently transcribed from *bldG* is *sco3550*, which codes for a putative RNA helicase. Interestingly, *bldG* and *sco3550* share common promoter regions suggesting that the expression of the putative RNA helicase and BldG could involve a coordinate control mechanism (Stoehr 2001). Additionally, *bldG* transcripts have several predicted hairpin structures that may require RNA helicase for unfolding. It is therefore speculated, although currently without any supporting evidence, that SCO3550 controls BldG activity directly or indirectly at the transcriptional or translational level. Structurally, BldG is related to RsbV and SpoIIAA, anti-anti-sigma factors of *B. subtilis.* BldG shares 40% identity, 60% similarity, and 26% identity, 56% similarity to RsbV and SpoIIAA, respectively (Bignell et al. 2000). The product of its downstream gene, ORF3, is also closely related to anti-sigma factors RsbW and SpoIIAB from *Bacillus*. (Bignell et al. 2000). SpoIIAA/SpoIIAB and RsbV/RsbW, in addition to several other proteins (including ribosomal or ribosome associated nucleoside triphosphate binding proteins) regulate sigma factors involved in the expression of genes required for sporulation and general stress response, respectively (Dufour and

Haldenwang 1994; Voelker et al. 1995; Pan et al. 2001; Scott and Haldenwang 1999; Zhang et al. 2001). A similar number and types of proteins could also control the activity/expression of BldG and these may include RNA helicases.

1.3. RNA Helicases

RNA helicases are enzymes that unwind duplex oligonucleotides into single strands in a nucleoside triphosphate (NTP) dependent fashion (Caruthers and Mckay 2002). They normally bind to the free single stranded end of duplexes and unidirectionally or bidirectionally disrupt hydrogen bonds in duplexes. RNA helicases are ubiquitous, found in all forms of life including viruses, and are encoded in up to 2% of the open reading frames in the genomes of most organisms (Lander et al. 2001; Silverman et al. 2003; Linder 2004). In the yeast *Saccharomyces cerevisiae*, over 120 open reading frames express putative helicases or helicase related proteins (Shiratori et al. 1999), while at least 10 putative RNA helicases were identified in *S. coelicolor* (Bentley et al. 2002). Despite their universal occurrence, the specific cellular function of most helicases is unknown. All characterized RNA helicases hydrolyze nucleoside triphosphate, with some using more than one kind of NTP as a substrate, and this activity is enhanced by the addition of RNA or DNA (de la Cruz et al. 1999; Jankowsky et al. 2000).

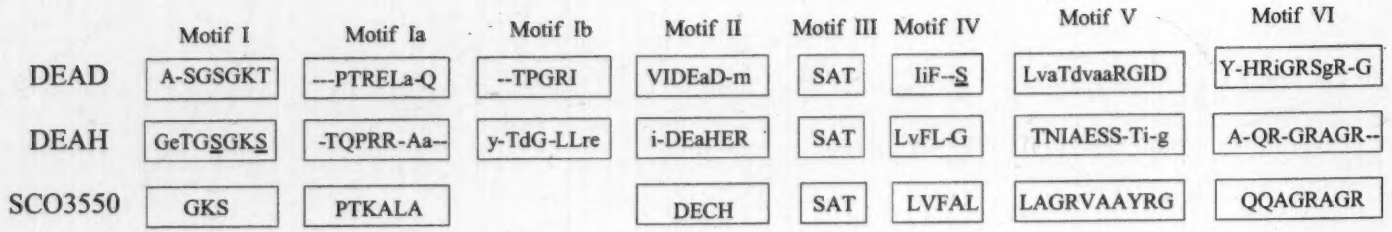
Helicases are classified into three large superfamilies and two smaller families by comparison of amino acid sequence homology (Gorbalenya and Koonin 1993). Among the three large superfamilies (SF), members of SF3 have three conserved motifs, whereas most members of SF1 and SF2 have between 7 to 9 motifs (Tanner et al. 2003; Rocak and Linder 2004; Tuteja and Tuteja 2004). The high degree of conserved organization

and sequence similarity in the conserved motifs suggests a common ancestor for genes coding for helicase proteins. Unlike the core region, the carboxyterminal domains of helicases are characterized by a high degree of sequence and length variability. It is hypothesized that variable carboxyterminal regions of helicases assign their specific functions by either providing substrate specificity or facilitating interactions within a protein complex (Tuteja and Tuteja 2004).

Members of SF2 are classified into three subfamilies by considering amino acid sequence variation in motif II, the Walker B motif (Walker et al. 1982). These subgroups consist of the DEAD-box, DEAH-box and the DExH-box RNA helicases (Fig.1.1). The DEAD-box RNA helicases can be distinguished from DEAH/DExH-box RNA helicases by having HRXGRXGR as a consensus sequence for motif VI whereas the latter have QRXGRXGR. Additionally, the DEAD-box helicases use mainly ATP as a substrate while the other subgroups often use more than one type of NTPs as substrate (Rocak and Linder 2004).

There are nine known RNA helicase motifs. Motif I (AXXGXGKT), also called Walker A motif (Walker et al. 1982), binds the β and γ phosphates of an ATP molecule through the lysine (K) residue. A point mutation caused by the replacement of the lysine by an uncharged amino acid asparagine in a prototypal DExD/H-box protein, mammalian initiation factor 4A (eIF4A), affected ATP binding of this protein (Pause and Sonenberg 1992). As described above, motif II is the specific form of the ATPase B motif (Walker et al. 1982). The negatively charged aspartate residue of motif II, is likely to interact with Mg^{2+} in an ATP/Mg^{2+} complex (Fry et al. 1986). A mutation in motif II of eIF4A was

Figure 1.1. Comparison of conserved motifs of the subfamilies of helicase superfamily II (SF2). The DExH subfamily is represented by SCO3550. In capital letters are the amino acids conserved at least 80% of the time while in small letters are those conserved 50%-79% of the time. The underlined serine (S) residues could be replaced with threonine (T). The – stands for any amino acid residue. Adapted from Tanner and Linder (2001).


Motif I
Motif Ia
Motif Ib
Motif II
Motif III
Motif IV
Motif V
Motif VI
DEAD
A-SGSGKT
---PTRELa-Q
--TPGRI
VIDEaD-m
SAT
IiF--S
LvaTdvaaRGID
Y-HRiGRSgR-G
DEAH
GeTGSGKS
-TQPRR-Aa--
y-TdG-LLre
i-DEaHER
SAT
LvFL-G
TNIAESS-Ti-g
A-QR-GRAGR--
SCO3550
GKS
PTKALA
DECH
SAT
LVFAL
LAGRVAAYRG
QQAGRAGR

shown to abolish ATP hydrolysis but permitted ATP binding, suggesting ATP hydrolysis as the function for this motif (Pause and Sonenberg 1992). Further mutational studies on vaccinia virus DExH-box RNA helicase, NPH-II, linked motif I and II to ATP hydrolysis (Gross and Shuman 1998). Motif III defined by TAT/SAT amino acid sequences is peculiar to the RNA helicases (Luking et al. 1998). Motif II and III can interact through hydrogen bond formation (Caruthers et al. 2000). Mutations in motif III freeze RNA helicase activity but allow ATP binding and hydrolysis (Pause and Sonenberg 1992; Pause et al. 1993). The motif therefore connects ATP binding and hydrolysis to conformational changes that are needed for helicase activity (Rocak and Linder 2004). Like motif III, motif VI is also unique to RNA helicases (Luking et al. 1998). Mutational analysis of motif VI from NPH-II of Vaccinnia virus (Gross and Shuman 1998), and eIF4A of yeast (Pause et al. 1993), assigned ATP binding and hydrolysis as the function to this motif. Recently, an additional motif, the Q motif (not shown in Fig. 1.1) has been described in eIF4A (Tanner et al. 2003). This motif contains 9 amino acids including a single invariant glutamine (Q), hence the name Q motif. A highly conserved phenylalanine occurs further upstream of the Q motif in eIF4A, which like the Q motif, is indispensable to the survival of the organism. The Q-motif is exclusively described for the DEAD-box RNA helicase subfamily and both the Q motif and the upstream phenylalanine are shown to be a further check for ATP binding and hydrolysis where an exposed Q motif may act as a limiting step for ATP binding and thus serves to regulate enzymatic activity (Tanner et al. 2003). The remaining motifs, Ia, Ib, IV, and V, are believed to be involved in RNA binding although there is less biochemical evidence to support this proposal (Rocak and Linder 2004).

1.4. Cellular functions of RNA helicases

1.4.1. Transcription

RNA helicases regulate several biological processes involving RNA metabolism and there is evidence that some may function without their unwinding activities (Fuller-Pace 2006). They may be involved in transcription of genes by stabilizing nascent transcripts or releasing completed transcripts from the template (Eisen and Lucchesi, 1998). A *Drosophila maleless* DEIH-box protein, Mle (maleless), and its homologue from Hela cells, RNA helicase A (RHA), have been shown to be involved in transcription. Nakajima et al. (1997) noted that RHA serves as a bridge between CREB-binding protein (CBP), an acetyltransferase enzyme that activates transcription, and RNA polymerase II (pol II), suggesting its involvement in the regulation of transcription. The overlapping region between the N-terminal domain and the core helicase domain was shown to associate with Pol II. The most N-terminal portion of the N-terminal domain from the helicase domain was shown to interact with CBP. Interestingly, mutations that silenced RNA helicase activity of RHA decreased CBP-dependent transcription. It has therefore been suggested that RHA may not only serve as a bridge between CBP and pol II, but may also act as a DNA helicase and remodel chromatin structure around the target promoters to facilitate transcription (Fuller-Pace 2006). In line with this, RHA had been shown to interact with a dsDNA and topoisomerase IIα required for the cycle of chromosome condensation and decondensation (Zhou et al 2003). In addition, RHA and its homologue, a bovine nuclear DNA helicase II, had been shown previously to possess both DNA and RNA helicase activities in vitro (Lee et al. 1997; Zhang and Grosse 1994). RHA also interacts with p65, a member of nuclear factor κB (NF- κB) (Tetsuka et al.

2004). NF- κB is an inducible cellular transcription factor that controls a large number of cellular and viral genes. RHA was shown to promote NF- κB dependent reporter gene expression induced by p65 and some other NF- κB -activators. Elimination of ATP binding activity of RHA abolished NF- κB dependent reporter gene expression induced by the activators. The above observations suggest that RHA mediates transcriptional activity of NF- κB (Tetsuka et al. 2004).

The *Drosophila* Mle protein plays similar roles in transcription as its mammalian homologue. To ensure dosage compensation, a process that equalizes transcription activity between the sexes, *Drosophila* uses two non-coding RNAs (*roX1* and *roX2*) and at least six protein regulators, which include Mle. Binding of Mle to a short upstream genomic region of the *roX2* gene has been shown to stimulate transcription of the gene downstream of *roX2* (Lee et al 2004).

While Mle and RHA are examples of helicases that play a role in transcription initiation, NPH-II, a prototypic DExH-box protein from vaccinia virus, appears to play a role in transcription termination. Conditional mutations of NPH-II, culminated in the production of abnormally long RNA transcript but initiation occurred accurately even though it was at a reduced frequency. It was therefore suggested that, NPH-II was required for efficient transcript termination (Gross and Shuman 1996).

The DEAD-box subfamily of superfamily 2 is not left out in the transcriptional role of RNA helicases as transcription is well-studied with the DEAD-box protein, mammalian p68 (Ford et al. 1988; Hirling et al. 1989). p68 activates transcription. As an activator of transcription, p68 localizes principally in the cell nucleus and is found to associate with a transcriptional coactivator p300, or its paralogue, CREB-binding protein

(CBP). This association was confirmed by coimmunoprecipitation assays (Goodman and Smolik 2000; Janknecht 2002; Rossow and Janknecht 2003). Additional support for the role of p68 as a transcriptional activator is the observed interaction between p68 and RNA polymerase II (poI II) (Rossow and Janknecht 2003). The p68 site of interaction with pol II is entirely different from that used to interact with p300/CBP, suggesting that p68 has the ability to associate simultaneously with both (Rossow and Janknecht 2003) and acts as a bridging factor similar to the DEAH-box protein, RHA. Furthermore, p68 stimulates the TPA oncogene responsive unit (TORU) promoter activity and an inhibition of CBP/p300 function by adenoviral protein E1A eliminates TORU promoter activation by p68, suggesting that p68, p300/CBP and RNA pol II work synergestically to trigger gene expression (Rossow and Janknecht 2003).

Another DEAD-box protein involved in transcription is DP103. It is a 825 amino acid protein, which like p68, binds to nuclear receptor proteins (Yan et al. 2003). It was identified as a result of its interaction with Epstein-Barr virus nuclear antigens, EBNA2 and EBNA3C (Grundhoff et al. 1999). Additionally, DP103 interacts with the orphan nuclear receptor steroidogenic factor 1 and represses its transcriptional activity (Yan et al. 2003). The steroidogenic factor 1 is required for the development and function of the endocrine and reproductive system (Ou et al. 2001; Parker and Schimmer 1997; Sadovsky and Dorn 2000).

Another DEAD-box RNA helicase, DP97, had been isolated from a mammalian cell line and was shown to interact with estrogen receptors (ER) and other nuclear receptors in a ligand-dependent manner (Rajendran et al. 2003). When endogenous cellular DP97 is knocked out by either RNA interference or antisense DP97, extradiol-

ER-stimulated genes are overexpressed, whereas genes inhibited by estradiol-ER are underexpressed. This suggests that DP97 intensifies the repression of estradiol-ER-regulated genes (Rajendran et al. 2003).

1.4.2. Translation

In many organisms, the process of protein synthesis has been shown to involve RNA helicases, which may be needed for unwinding secondary structure in the 5' non-coding region of mRNAs to assist ribosome binding, or may be involved in ribosome assembly (Pause and Sonenberg 1992). Translation initiation involving RNA helicases has been studied intensively with the eukaryotic translation factor 4A (eIF4A), a prototypical DEAD-box RNA helicase. The process begins with the assembly of three initiation complexes, 43S, 48S, and 80S, in a stepwise manner and each step is catalyzed by an initiation factor (Korneeva et al. 2005). Formation of the 48S complex by the recruitment of mRNA to the 43S complex is the rate-limiting step and it is catalyzed by the eIF4 initiation factor complex. In mammals, the eIF4 consists of eIF4A, a 46 kDa ATP-dependent RNA helicase that exists in a complex known as eIF4F with a 25 kDa protein eIF4E, and a 185 kDa protein eIF4G (Korneeva et al. 2005; Edery et al. 1983; Grifo et al. 1983). eIF4E forms the link between eIF4F and the mRNA by binding to the mRNA cap while eIF4G binds other factors involved in the recruitment of the mRNA and anchors them to the 40S ribosomal subunit through binding to eIF3. The 40S subunit and eIF3 are components of 43S complex. eIF4G contains two eIF4A binding sites that interact directly with the N-terminal domain of eIF4A, where the conserved ATP and the oligonucleotide binding motifs are found (Korneeva et al. 2005). Another member of the eIF4 initiation complex is eIF4B, an 80 kDa protein that enhances ATPase and helicase

activities of eIF4A in several processes, even though there is no evidence of a direct physical interaction between them. The stimulatory activity of eIF4B is enhanced further in the presence of eIF4H, another component of the eIF4 complex (Rozen et al. 1990; Richter et al. 1999; Rogers et al. 1999). Apart from a biochemical connection between eIF4A and eIF4B, Coppolecchia et al. (1993) observed that multiple copies of eIF4B could suppress a temperature sensitive eIF4A mutation in yeast, indicating a genetic link between them.

The first step in the initiation of translation requires bringing of eIF4A close to the 5' unstranslated region (UTR) of the mRNA via the cap-binding protein eIF4E. eIF4B also attaches itself to a subunit of eIF3 close to 5' UTR secondary structure and enhances the activity of eIF4A (Methot et al. 1996). eIF4G probably forms a bridge between eIF4A and eIF4B by binding to eIF3. Unwinding of the secondary structure in the 5' UTR is then carried out by eIF4A, allowing for the binding and scanning of the small ribosomal subunit in order to initiate translation at the designated start codon (Svitkin et al. 2001; Silverman et al. 2003).

Two homologues of eIF4A in mammals, eIF4AI and eIF4AII , and two others, Tif1 and Tif2 in yeast, have been previously described (Nielsen and Trachsel 1988; Linder and Slonimski 1989). The disruption of the *tif* genes in yeast is detrimental to the growth of the cell since protein synthesis is abolished (Linder and Slonimski 1989; Prat et al. 1990). The yeast *tif* double mutants are rescued upon the addition of exogenous eIF4A (Blum et al. 1989), suggesting that the products of the *tif* genes may play similar roles in the cell as eIF4A. Another DEAD-box protein from *Saccharomyces cerevisiae*, DED1, has also been implicated in translation initiation. Using biochemical and genetic analysis,

Chuang et al (1997) observed that translation was drastically diminished in the conditional mutants of *ded1*. They proposed that DED1 may play similar role in translation as elF4A.

Apart from the yeast proteins mentioned above, mammalian RHA (described above to have a role in regulation of transcription, section 1.4.1) is also implicated in translation. Recent work by Hartman and others revealed that RHA can recognize a structured 5'-terminal post-transcriptional control element (PCE) of a retrovirus and a human intronless growth-control gene, *JUND*. RHA was shown to associate with PCE RNA in the nucleus and cytoplasm, to promote polyribosome association and to be necessary for its efficient translation (Hartman et al. 2006).

1.4.3. Ribosome biogenesis

In addition to translation and transcription, RNA helicases also play roles in ribosome biogenesis. Ribosome biogenesis in *E. coli* involves three ribosomal RNAs (rRNAs), 23S, 16S, and 5S, produced from a common rRNA precursor, and 54 ribosomal proteins. Interplay of 23S and 5S rRNAs and 33 ribosomal proteins gives rise to a mature 50S ribosomal subunit. On the other hand assembly of the 16S rRNA with the remaining 21 ribosomal proteins produces the mature 30S subunit (Srivastava and Schlessinger 1990; Nierhaus 1991). The assembly of the ribosomal subunits in vitro takes a longer time to complete than their assembly within the cell, suggesting that some catalytic factors are missing in vitro (Charollais et al. 2003). This suggests that there are most likely some uncharacterized proteins involved in ribosome biogenesis.

Two DEAD-box proteins from *Escherichia coli*, CsdA and SrmB, participate in the synthesis of the 50S ribosomal subunit. CsdA (cold-shock DEAD-box protein A),

formerly called DeaD, was discovered by virtue of its ability as a multicopy suppressor of a rps mutant that lacks normal expression of ribosomal protein S2 (Toone et al. 1991). SrmB was also identified by its ability to restore wild type phenotype to a temperature-sensitive mutant of a gene coding for ribosomal protein L24 (Nishi et al. 1988). Further evidence supporting involvement in ribosome biogenesis came from the observation that at temperatures below 20^0C, *E. coli csdA* and *srmB* mutants have reduced quantities of the 50S ribosomal subunit but that new particles accumulate and sediment around 40S (Charollais et al. 2003; Charollais et al. 2004). Analysis of the ribosomal protein and rRNA content of the 40S macromolecules confirmed that they resulted from incomplete assembly of the 50S subunit (Charollais et al. 2003; Charollais et al. 2004). As a result, SrmB and CsdA were implicated in the synthesis of 50S subunit. The 40S macromolecule obtained from a *srmB* mutant lacks L13, one of the five ribosomal proteins that is necessary for the early assembly of 50S ribosomal subunit. SrmB is therefore thought to catalyze early stages of 50S synthesis (Charollais et al. 2003), whilst CsdA is thought to act later in the processes (Charollais et al. 2004). The mutants of the *srmB* gene grow slowly at low temperature, yet upon overexpression of CsdA, the wild type phenotype is restored in these mutants, indicating some functional overlap between the two genes (Charollais et al. 2003; Charollais et al. 2004).

1.4.4. Remodeling of ribonucleoproteins

Some RNA helicases may act as RNPase (have the ability to dislodge protein from protein-RNA complex independent of RNA unwinding). This activity has been described for NPH-II, a DExH RNA helicase. NPH-II (nucleoside triphosphate phosphorylase II) from vaccinia virus was the first RNA-dependent ATPase to be characterized (Paoletti et

al. 1974; Paoletti and Moss 1974), and it is considered to be the prototype of the DExH-box RNA helicases (Gross and Shuman 1996). The RNPase activity of NPH-II has been tested in vitro on two ribonucleoproteins: tryptophan RNA-binding attenuation protein (TRAP) in association with a specific 53-nucleotide cognate RNA, and an exon junction complex (EJC) deposited on a mRNA as a result of splicing (Antson et al. 1999; Babitzke and Gollnick 2001; Le Hir et al. 2003). NPH-II dissociates the two complexes in an ATP dependent-fashion although to a lesser extent in the EJC complex. The dissociation occurs in the absence of RNA unwinding activity, suggesting no correlation between RNPase and helicase activities (Fairman et al. 2004). A comparable result was obtained on the EJC complex with a DEAD-box protein, DED1, however, DED1 could not disrupt the TRAP complex (Fairman et al. 2004; Jankowsky et al. 2000), indicating different ribonucleoprotein target requirements for different types of RNA helicases. Further proof to demonstrate that DExD/H-box proteins can dislodge proteins from ribonucleoproteins was described by Jankowsky et al. (2001). They showed that NPH-II removed U1A protein, a component of spliceosomal machinery, from a complex of ribonucleoprotein.

1.4.5. RNA decay

RNA helicases are also required for RNA degradation. A DEAD-box RNA helicase RhlB is a component of the *Escherichia coli* RNA degradosome and has been shown to enhance RNA degradation (Py et al. 1996). Other members of the degradosome are: a glycolytic enolase enzyme whose role in RNA metabolism is unknown; a polynucleotide phosphorylase (PNPase) that possesses 3' to 5' exoribonucleolytic activity; RNaseE that acts as an endoribonuclease; and a multifunctional protein, DnaK, which participates in the heat shock response (Miczak et al. 1996; Py et al. 1996). RNase E

triggers decay of mRNA by endoribonuclease digestion at specific cleavage sites. The degradation products of RNase E are further broken down into nucleotides by PNPase and other exoribonucleases. Previous experiments indicated that RhlB facilitates the degradation process by enhancing the activity of PNPase in an ATP-dependent manner (Py et al. 1996). An alternative role for RhlB in the degradosome was described by Khemici et al. (2005). They showed that a deletion of the gene that codes for RhlB stabilizes a *lacZ* mRNA and other messages transcribed by bacteriophage T7 RNA polymerase. Their study further revealed that RhlB facilitates endoribonucleolytic cleavage by RNase E (Khemici et al. 2005). Recently, Khemici et al. (2004) showed that RNase E has at least two sites for binding RNA helicases: a site for binding to RhlB, a known member of the degradosome, and a different site for binding to three other DEAD-box proteins, SrmB, RhlE, and CsdA. As stated above, CsdA and SrmB have previously been shown to be involved in the synthesis of 50S ribosomal subunits (Charollais et al. 2003; Charollais et al. 2004). RhlE has no known role but has been found to associate with poly(A) polymerase I (Ohmori 1994; Raynal and Carpousis 1999). RNA decay studies in vitro showed that either RhlE or CsdA could interchangeably replace RhlB in enhancing RNA degradation (Khemici et al. 2004), implying that RNase E could be promiscuous in the choice of RNA helicase for its activity. In line with this, CsdA, was co-purified with the components of the degradosome (Prud'homme-Genereux et al. 2004).

A putative NTP-dependent RNA helicase, SuV3, with a degenerate DExH-box (DEIQ) was found to be part of the yeast mitochondrial 3' to 5' exoribonuclease that degrades group I introns. There was about 50 fold increase in the accumulation of the

spliced group I intron ω of the mitochondrial 21S rRNA gene when *suv3* was disrupted (Margossian et al. 1996). An accumulation of group I introns is fatal to the cell since the catalytic activities of the intron may affect transcription, translation, and RNA processing in the mitochondria (Margossian et al. 1996).

1.4.6. mRNA splicing

The splicing of the nuclear precursor mRNA (pre-mRNA) is a complex process involving sequential recruitment of five small nuclear ribonucleoproteins (snRNPs), U1, U2, U5, and U4/U6, in addition to several other proteins onto the pre-mRNA (Will and Luhrmann 1997). In complex, the snRNPs and the non-snRNP proteins are referred to as the spliceosome. In vitro, the splicing process proceeds with the formation of the spliceosome followed by two sequential esterification reactions to remove introns, release of the mature mRNA and the reformation of the snRNPs (Silverman et al. 2003). At least eight DExD/H-box proteins are implicated in this process and are required for maintaining the progressive continuity of the spliceosome assembly most likely by modifying RNA-RNA or RNA-protein interactions (Silverman et al. 2003). One of the earliest DExD/H-box proteins described to be involved in this process is the human 56kDa DECD-box RNA helicase, UAP56, a homologue of Sub2 from yeast. UAP56 was first identified due to its interaction with U2AF65, a splicing factor that is required for the entry of U2 snRNP into the spliceosome. U2AF65 specifically interacts with the polypyrimidine tract of the pre-mRNA. UAP56 also catalyzes the binding of the U2 snRNP to the pre-mRNA intron-exon junction opposite to the position where U1 snRNP is attached (Fleckner et al. 1997; Kistler and Guthrie 2001). The homologue of UAP56 from yeast, Sub2, was identified through complementation of a mutation in the *brr1* gene

that codes for a splicing factor involved in snRNP biogenesis (Kistler and Guthrie 2001). Additionally, Sub2 was identified by complementation studies in the fission yeast *Schizosaccharomyces pombe* (Libri et al. 2001). A mutation in the *sub2* gene culminates in the accumulation of an intermediate that contains U2 snRNP which can be chased into functional spliceosome (Libri et al. 2001). Like the mammalian homologue, Sub2 is required for the addition of U2 snRNP to the spliceosome (Libri et al. 2001; Zhang and Green 2001) and may even be involved in events prior to spliceosome formation (Zhang and Green 2001). Mud2 is the yeast homologue of the human splicing factor, U2AF65. The deletion of *mud2* partially eliminates the necessity of Sub2 in the spliceosomal assembly process (Kistler and Guthrie 2001). This implies that in the *mud2* mutant background, the requirement for Sub2 could be dispensed with (Kistler and Guthrie 2001).

Another RNA helicase required for the early stage of the splicing process is Prp28 (for pre-mRNA processing), a member of the penta-snRNP. The penta-snRNP complex comprises the initial assembly of the five snRNPs as well as non-snRNP proteins (Stevens et al. 2002). Prp28 was originally identified during a genome-wide search for DEAD-box proteins in *Saccharomyces cerevisiae* (Chang et al. 1990). A mutation in *prp28* results in a cold-sensitive phenotype and a blockage in the initial step of splicing at this restrictive temperature (Strauss and Guthrie 1991; Strauss and Guthrie 1994). Prp28 might not require helicase activity to function since purified Prp28 fails to unwind RNA duplexes in vitro. However, there is a possibility that it may require cofactor(s) to facilitate this activity or may work solely on a physiologically relevant RNA substrate (Strauss and Guthrie 1994). Alternatively, the possibility that it can only

perform a minimum level of helicase activity in vivo, as noted for some other RNA helicases, cannot be ruled out (Silverman et al. 2003). Interestingly a disruption of the *prp8* gene, which codes for a splicing factor that associates with U5 snRNP, restores wild type phenotype to *prp28* mutant suggesting that Prp8 may be involved in the regulation of Prp28 (Strauss and Guthrie 1991), perhaps by functioning as its cofactor. Staley and Guthrie (1999) used genetic analysis to show that Prp28 mediates the switch of U1 for U6 at the 5' splice site in the pre-mRNA. This discovery led to the assignment of the U1 snRNA-5' splice site RNA duplex (U1/U6 duplex) as a likely target for Prp28 in vivo (Silverman et al. 2003). Interestingly, like the *prp8* gene, a mutation in a gene that codes for U1 snRNP protein also suppresses a mutation in *prp28* (Zhang and Rosbash 1999; Chen et al. 2001). This finding confirms a link between Prp28 and U1 and suggests a possible role of the U1 snRNP as a cofactor for Prp28.

A DExH-box RNA helicase from yeast, Brr2, catalyzes the dissociation of the U4/U6 duplex in an ATP-dependent manner (Raghunathan and Guthrie 1998). Apart from unwinding the U4/U6 duplex, it may play an additional role in splicing since it is an integral snRNP protein (Silverman et al. 2003). Brr2 interacts with several splicing factors such as Prp8 and two helicases in the splicing pathway, Prp2 and Prp16, suggesting its involvement in the sequential interactions of the DEAH-box helicases within the splicing complex (van Nues and Beggs 2001). A homologue of Brr2 from human HeLa cells, U5-200kD, has also been shown to unwind U4/U6 RNA duplexes in vivo (Laggerbauer et al. 1998). The splicing process is continuous since pre-mRNAs are produced constantly in the cell, snRNPs must therefore be regenerated to permit continuation of the cycle necessary for pre-mRNA splicing to occur. An RNA binding

protein Prp24 has been described in yeast that controls reannealing of U4 and U6 transcripts after their dissociation was catalyzed by Brr2 (Raghunathan and Guthrie 1998). When Prp24 was absent from an in vitro spliceosomal reaction mix, U4 and U6 snRNPs accumulated and ultimately splicing was impeded. Subsequently, addition of purified Prp24 to the mix restored the splicing process and prevented the accumulation of U4 and U6 snRNPs. (Raghunathan and Guthrie 1998).

At least three DEAH-box RNA helicases from yeast including Prp2, Prp16 and Prp22 are thought to regulate the two transesterification reactions in splicing (Schwer 2001). Prp2 drives the initial transesterification reaction in an ATP-dependent manner (Kim et al. 1992; Kim and Li 1993). Prp16 catalyzes the second transesterification reaction and this also requires ATP binding and/or hydrolysis (Schwer and Guthrie 1991). The third DEAH-box RNA helicase mentioned above, Prp22, was shown to play at least two roles in the splicing of yeast pre-mRNA. It is required for the release of mRNA from the spliceosome using ATP-dependent helicase activity (Schwer and Meszaros 2000) and it also prevents aberrant mRNA formation by discriminating against aberrant introns using both unwinding and ATPase activities (Mayas et al. 2006).

Group I and II introns are found in some mitochondria, chloroplasts, pre-rRNAs, pre-tRNAs and pre-mRNAs. These introns are capable of self-splicing in vitro but may require protein for efficient splicing within the cell (Silverman et al. 2003). CYT-19, a DEAD-box protein from the fungus *Neurospora crassa,* and CYT-18, a tyrosyl-tRNA synthetase are required for the correct splicing of mitochondrial group I introns. CYT-18 stabilizes the catalytically active RNA structure whereas CYT-19 resolves the kinetically-trapped inactive RNA conformers (Akins and Lambowitz 1987; Mohr et al. 2002).

Mitochondrial splicing proceeds in the absence of active CYT-19, however some 5' and 3' processing reactions are blocked suggesting that CYT-19 may function as an RNA chaperone, which destabilizes misfolded RNAs (Mohr et al. 2002). Recently, a DEAD-box protein from *S. cerevisiae*, Mss116, was shown to act as an RNA chaperone in the splicing of mitochondrial group I and II introns (Huang et al. 2005), therefore a range of DEAD-box proteins may be required as RNA chaperones for these processes (Huang et al. 2005).

1.4.7. Formation of small interfering RNAs

Cellular functions including gene silencing and developmental regulation of gene expression require small interfering RNAs (siRNAs) derived from double stranded RNA (dsRNA) precursors whose processing requires members of the DExD/H-box protein family (Zamore 2001; Hannon 2002). siRNAs are approximately 22 nucleotides long and are complementary to the transcript of the gene that they suppress (Hammond et al. 2000). Their function is to guide and instruct multi-component nucleases to destroy specific mRNAs (Hammond et al. 2000). A DExH-box protein implicated in this process is Dicer. Its name stems from the fact that it is able to produce equal sized small RNAs from the precursor dsRNA (Bernstein et al. 2001). Dicer is evolutionary conserved in worms, flies, plants, fungi, and mammals and has three distinct functional domains: a DECH motif at the N-terminal region, an RNase III motif at the C-terminus, and a region of homology to the RDE1/QDE2/ARGONAUTE family of proteins that has been shown to be genetically linked to RNA interference (Tabara et al. 1999; Catalanotto et al. 2000; Bernstein et al. 2001). It was proposed initially that Dicer unwound the precursor dsRNA in an ATP-dependent fashion before cleavage by the RNase III domain (Bernstein et al.

2001). Different research groups have, however, demonstrated that the ATPase activity of Dicer was independent of the RNase activity (Provost et al. 2002; Zhang et al. 2002). The helicase activity of Dicer in the processing of siRNAs has thereby been speculated to be involved in the transfer of siRNAs to downstream components, such as the RNA-induced silencing complex (RISC). In support of this, Dicer has been shown to interact with an Argonate protein found in the Drosophila RISC complex (Hammond et al. 2001). Additionally, Dicer forms a complex with a dsRNA binding protein in *Caenorhabditis elegans*, which most likely facilitates the delivery of dsRNA precursors to Dicer to be processed (Tabara et al. 2002).

1.4.8. mRNA transport

RNA helicases are implicated in the transfer of mRNAs produced in the nucleus to the cytoplasm for their translation. From the work of Gross and Shuman that unveiled a possible role for NPH-II in transcription termination (see section 1.4.1), it was observed that there was accumulation of the abnormally long transcripts where they were synthesized (Gross and Shuman 1996). The deduction made from this observation was that in the absence of functional NPH-II, transcripts may not be made available to the RNA extrusion machinery (Gross and Shuman 1996). Another RNA helicase involved in mRNA transport is Sub2. The idea that Sub2, a DECH-box RNA helicase, might be involved in the transport of mRNA arose from its genetic interaction with Yra1p (Yeast RNA-RNA annealing protein 1), an indispensable mRNA export factor (Strasser and Hurt 2001). Protein interaction studies have shown that Yra1p also interacts with Mex67p, another nuclear export factor, whose homologues are found in humans and *C. elegans* (Segref et al. 1997; Strasser and Hurt 2000). In both cases mutations in the *yra1p*

and *mex67p* genes led to the accumulation of poly(A)$^+$ RNA in the nucleus under restrictive growth conditions (Segref et al. 1997; Strasser and Hurt 2000). Disruption, or overexpression, of the *sub2* gene culminated in the accumulation of poly(A)$^+$ RNA as found in the *yra1* and *mex67* mutants (Jensen et al. 2001; Strasser and Hurt 2001). Also, inhibition of mRNA transport by an excess amount of Sub2 suggests that the process must require an optimal concentration of Sub2 to function. Thermosensitive *sub2* mutants showed normal export of mRNA at the permissive temperature but a drastic accumulation of poly(A)$^+$ RNA in the nucleus within a short period of transfer to the restrictive temperature. The quick poly(A)$^+$ RNA accumulation suggests a direct involvement of Sub2 in mRNA export. Sub2 was implicated in mRNA transport but not the transport of other types of RNAs (Strasser and Hurt 2001). As previously described, a deletion of *mud2* makes Sub2 dispensable for pre-mRNA splicing (Kistler and Guthrie 2001). Poly(A)$^+$ RNA accumulates in *sub2* and *mud2* double mutants although to a lesser extent than what was observed for thermosensitive *sub2* mutants (Strasser and Hurt 2001). Consequently, a *mud2* deletion cannot therefore fully substitute for the role of Sub2 in mRNA export as observed in pre-mRNA splicing (Strasser and Hurt 2001). Sub2 and Mex67p have the same binding site in Yra1p and these proteins are shown to compete with each other for the binding site (Strasser and Hurt 2001). Since Mex67p is confined to the nuclear pore while Yra1p and Sub2 are located in the nucleoplasm, Sub2 may function to recruit Yra1p to the mRNA and subsequently be displaced from Yra1p by binding of Mex67p (Strasser and Hurt 2001). This suggests an early role of Sub2 in mRNA transport out of the nucleus. More evidence to support Sub2 as an early mRNA transporter comes from the accumulation of an intronless transcript, *HSP104,* in a *sub2*

mutant (Jensen et al. 2001). *HSP104* transcripts were observed to be at or near their site of transcription in the *sub2* mutant. Additionally, Sub2 interacts genetically and functionally with Rad3p, an RNA polymerase II-associated DNA/DNA:RNA helicase, and with Rrp6p, a nuclear RNA exosome component that is known to retain mRNAs at the transcription sites (Jensen et al. 2001). Mutational studies with UAP56, a human homologue of Sub2, and Aly, a human homologue of Yra1p, showed a comparable effect on mRNA transport (Luo and Reed 1999). Overexpression of UAP56 inhibits export of mRNA as well as the recruitment of Aly to the spliced mRNA-protein complex (mRNP). Additionally, the disruption of the interaction between Aly and UAP56 prevents the recruitment of Aly to the spliced mRNP. These results suggest that the splicing factor UAP56 links splicing and export machineries by recruiting Aly to the spliced mRNA, and this further supports the findings that splicing of pre-messenger RNA and export of mRNA are coupled in the cell (Luo and Reed 1999; Zhou et al. 2000; Huang and Steitz 2001; Rodrigues et al. 2001).

1.4.9. Other functions

At least three known DExH-box RNA helicases are required for viral replication: NPH-II from vaccinia virus, NS3 (non-structural protein 3) from hepatitis C virus, and the plum virus cylindrical inclusion (CI) protein. As stated earlier, NPH-II is also necessary for the early mRNA synthesis in the viral particle and could be involved in transcription termination and RNA transport (Gross and Shuman 1996). NPH-II has recently been shown to have RNPase activity in vitro (Fairman et al. 2004), and it is less processive on double stranded DNA (dsDNA) than dsRNA (Kawaoka and Pyle 2005). NS3, however, is highly processive on dsDNA substrates, whereas a low processivity on

dsRNA substrates is observed in the absence of a NS4A as a cofactor. Although hepatitis C virus is a RNA virus, the high level of processivity on dsDNA substrates suggests that NS3 may target the host DNA (Pang et al. 2002). NS3 is a proteolytic modified product of a polyprotein encoded by the 9.6 kb positive single stranded RNA genome of hepatitis C virus (Kwong et al. 2000). It is a multifunctional protein with a serine protease activity at the N-terminus as well as NTPase and RNA unwinding activities at the C-terminal region (Kwong et al. 2000). Accordingly, NS3 has four conserved amino acid motifs, including a DECH-box, which are common to those of the members of SF2 RNA helicases (Kim et al. 1997). Like NPH-II from vaccinia virus, NS3 plays a pivotal role in the replication cycle of the hepatitis C virus. Undoubtedly, it is currently an excellent target for drug design for the treatment of hepatitis C (Locatelli et al. 2002). The third RNA helicase mentioned above, the cylindrical inclusion protein from a positive stranded RNA virus, plum pox, is also characterized by a DECH-box (Lain et al. 1990). It displays all characteristics of a true RNA helicases: RNA binding, NTPase and RNA unwinding activities and is also predicted to be involved in viral replication (Fernandez et al. 1995).

Several DEAD-box proteins are implicated in RNA editing (posttranscriptional insertion and/or deletion of nucleotides in an mRNA molecule) in *Trypanosoma brucei* (Missel and Goringer 1994; Missel et al. 1997). A mutation in *hel61* that codes for a DEAD-box RNA helicase in *T. brucei* results in a low number of edited mRNAs (Missel et al. 1997). There are also many instances of a requirement for RNA helicases in cell differentiation and developmental processes. For instance, it is believed that RNA helicases are probably involved in the formation of germ cells (Luking et al. 1998).

Additionally, the DEAD-box RNA helicase, Me31B, from *Drosophila* is highly expressed during oogenesis (de Valoir et al. 1991).

1.5. SCO3550, a putative RNA helicase from *S. coelicolor*

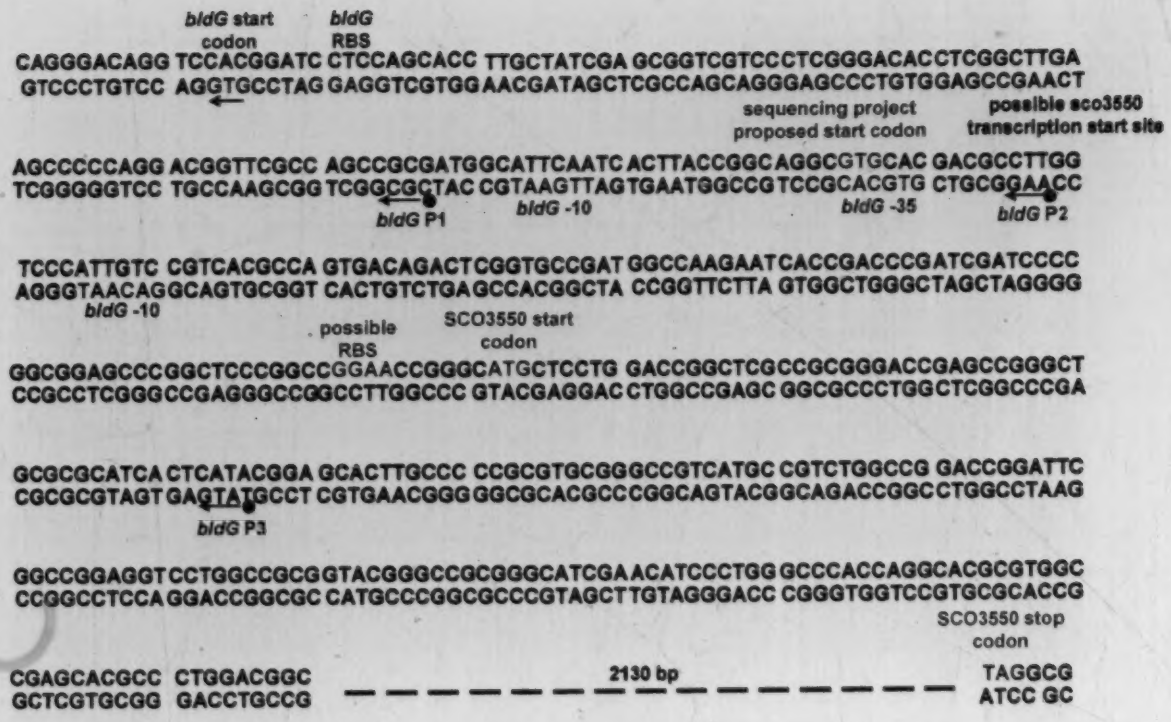
SCO3550 possesses seven conserved motifs common to RNA helicases (Fig.1.1). As mentioned earlier, *sco3550* is situated next to and divergently transcribed from *bldG*, whose gene product is a regulator of antibiotic synthesis. Interestingly, *bldG* transcripts have several predicted hairpin structures that may require RNA helicase for unfolding. Based on the prediction that *bldG* and *sco3550* may have common transcriptional regulatory pathway due to an overlapping promoter regions, it is speculated, although currently without any supporting evidence, that SCO3550 controls BldG activity directly or indirectly at the transcriptional or translational level. The numerous probable connections between SCO3550, a putative RNA helicase, and BldG, a major regulator of antibiotic synthesis and morphological differentiation, makes it an excellent protein to study. SCO3550 is probably an indispensable gene in the development of *S. coelicolor* as several attempts to construct its null mutant were unsuccessful (Stoehr 2001; Gislason 2005). The expression level of *sco3550* in the cell appears to be very low. No signal of *sco3550* mRNA was detected with S1 nuclease protection or primer extension, the two conventional methods used for studying gene expression levels. The transcripts were however detected by a reverse transcriptase polymerase chain reaction (RT-PCR), which showed that *sco3550* is expressed throughout the life of *S. coelicolor* with two peaks concomitant with vegetative growth and aerial hyphae formation (Stoehr 2001). On the basis of the occurrence of these peaks and the fact that *sco3550* disruption was possible in a *bldG* mutant but not the wild type

background, SCO3550 is speculated to play two distinct roles, one being the suppression of genes involved in sporulation during early growth of the cell to ensure cell viability and a switch of this function to another at the time of aerial hyphae formation (Stoehr 2001). Furthermore, by the use of RT-PCR coupled with primer walking, the more likely translational start site of *sco3550* has been located to an ATG start codon, downstream of the predicted GTG start codon assigned by the annotators of *S. coelicolor* genome project (Stoehr 2001). Further experiments indicated that SCO3550 may be expressed from the ATG and GTG start codons as isoforms. Western analysis of SCO3550 revealed two forms of the protein (Gislason 2005). Moreover, the expression level of both isoforms were affected in a *bldG1DB* in-frame deletion mutant (Gislason 2005). These observations have led to the current belief that, during the earlier transcript localization study by Stoehr, in which the 5' end of the SCO3550 transcript was mapped to with a few bases of the GTG codon, a low signal corresponding to a full-length transcript initiating upstream of the GTG codon might have been missed. *sco3550* is therefore speculated to be expressed from two translational start sites, ATG and GTG start codons (Fig.1.2) corresponding to the smaller and the larger isoforms, respectively.

1.6. Project objective

Previous biochemical analysis with a maltose-binding protein (MBP) tagged ATG SCO3550 showed that the recombinant protein destabilized dsRNA in the presence or absence of ATP (Gislason 2005). This observation of ATP-independence is uncommon among the DExD/H-box proteins. To date, it has been described for only a few characterized RNA helicases (Boddeker et al .1997; Jones et al 1996). A concern was that the relatively large MBP-tag might affect the biochemical activity of the protein.

Figure 1.2. Putative translational start codons of *sco3550*. The putative GTG start codon was predicted by the annotators of the *S. coelicolor* genome project while the ATG start codon considered to be the more likely translational start codon, was identified in transcript localization studies using RT-PCR and primer walking. A possible ribosome binding site, GGAA, is located upstream of the ATG start codon. Also shown are the *bldG* translational start site and the promoter region. Adapted from Stoehr (2001).


bldG start codon
bldG RBS
CAGGGACAGG TCCACGGATC CTCCAGCACC TTGCTATCGA GCGGTCGTCCCTCGGGACACCTCGGCTTGA
GTCCCTGTCC AGGTGCCTAG GAGGTCGTGG AACGATAGCTCGCCAGCAGGGAGCCCTGTGGAGCCGAACT
sequencing project proposed start codon
possible sco3550 transcription start site
AGCCCCCAGG ACGGTTCGCC AGCCGCGATGGCATTCAATC ACTTACCGGC AGGCGTGCAC GACGCCTTGG
TCGGGGGTCC TGCCAAGCGG TCGGCGCTAC CGTAAGTTAG TGAATGGCCG TCCGCACGTG CTGCGGAACC
bldG P1
bldG -10
bldG -35
bldG P2
TCCCATTGTC CGTCACGCCA GTGACAGACT CGGTGCCGAT GGCCAAGAATCACCGACCCGATCGATCCCC
AGGGTAACAGGCAGTGCGGT CACTGTCTGA GCCACGGCTA CCGGTTCTTA GTGGCTGGGCTAGCTAGGGG
bldG -10
possible RBS
SCO3550 start codon
GGCGGAGCCCGGCTCCCGGCCGGAACCGGGCATGCTCCTG GACCGGCTCGCCGCGGGACCGAGCCGGGCT
CCGCCTCGGGCCGAGGGCCGGCCTTGGCCC GTACGAGGAC CTGGCCGAGC GGCGCCCTGGCTCGGCCCGA
GCGCGCATCA CTCATACGGA GCACTTGCCC CCGCGTGCGGGCCGTCATGC CGTCTGGCCG GACCGGATTC
CGCGCGTAGT GAGTATGCCT CGTGAACGGG GGCGCACGCCCGGCAGTACGGCAGACCGGCCTGGCCTAAG
bldG P3
GGCCGGAGGT CCTGGCCGCG GTACGGGCCGCGGGCATCGA ACATCCCTGG GCCCACCAGGCACGCGTGGC
CCGGCCTCCA GGACCGGCGC CATGCCCGGCGCCCGTAGCT TGTAGGGACC CGGGTGGTCCGTGCGCACCG
SCO3550 stop codon
CGAGCACGCC CTGGACGGC
GCTCGTGCGG GACCTGCCG
2130 bp
TAGGCG
ATCC GC

Additionally, it was thought that the 5' region missing from the ATG-version but present in the GTG-version might have influenced the final structure and enzymatic activity of the protein. The objective of my project was therefore to verify whether the predicted SCO3550 isoforms are both active forms of the protein. To address whether the maltose-binding protein tag affected the enzymatic activity of the ATGSCO3550, a histidine tagged ATGSCO3550 would be used to retest the biochemical activities of the protein. The biochemical activities would also be tested with MBP-ATGSCO3550 and MBP-GTGSCO3550 and the results compared.

CHAPTER 2:
MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. Bacterial strains, plasmids and growth conditions

2.1.1. *Escherichia coli* and *Streptomyces coelicolor* strains used

The list of the bacterial strains used in this work is shown in Table 2.1.

2.1.2. Plasmids and vectors used

The cloning vectors and the recombinant plasmids used for this work are listed in Table 2.2.

2.1.3. Growth and maintenance of *Escherischia coli* strains

Liquid *E. coli* cultures were grown in LB medium at room temperature, 30^0C or 37^0C on a rotating rack or on a shaker. Solid cultures were grown on LB agar plates at 37^0C. Where needed for plasmid selection, cultures were grown in the presence of 100 μg/mL ampicillin.

2.1.4. Preparation *of E. coli* glycerol stocks

Single colonies of individual *E. coli* strains picked from LB agar plates were used to inoculate 5 mL of LB broth containing the appropriate antibiotic(s) and were grown overnight on a rotating rack at 37^0C. Aliquots of the overnight cultures were then mixed with glycerol to a final concentration of 10%, flash frozen in dry ice and stored at -86^0C.

2.1.5. Growth and maintenance of *S. coelicolor* strains

Streptomyces coelicolor strains were grown in 5 mL 2 x YT broth (Sambrook et al. 1989) in universal bottles with a 1 inch spring coil. Aliquots of the overnight cultures were then used to inoculate 50 mL R2YE broth (Hopwood et al. 1985) in 250 mL flasks with a spring coil. Incubation was done at 30^0C on a shaker set at 200 rpm. Surface cultures of *S. coelicolor* were grown on R2YE agar. Where necessary for plasmid

Table 2.1. Bacterial strains used in this work

Bacterial strain	Genotype	Reference/source
E. coli		
BL21(DE3)	F⁻ *ompT hsdS*$_B$($r_B^- m_B^-$) *gal dcm* (DE3)	Stratagene
BL21(DE3)pLysS	F⁻ *ompT hsdS*$_B$($r_B^- m_B^-$) *gal dcm* (DE3) pLysS (Cm^R)	Stratagene
DH5α	F⁻,ϕ80*lacZΔM15*Δ(*lacZYA-argF*)*U169*,*deoR recA1, hsdR17*(*rk-mk*)*phoA*, *supE44*, λ⁻ *thi-1 gyrA96, relA1*	Hanahan (1983)
ET12567	F-, dam13::Tn9, *dcm6*, *hsdR*, *recF143*, *zjj202*::TN10, *galK2*, *galT22*, *ara14*, *lacY1*, *xy15*, *leuB6*, *thi1*, *tonA31*, *rpsL136*, *hisG4*, *Tsx78*, *mtl 1*, *glnV44*	Macneil et al. 1992; Gift from D. MacNeil, Merck Sharp and Dohme Research Laboratories
S. coelicolor		
M600	SCP1⁻, SCP2⁻	Chakraburtty and Bibb (1997); Gift from M. Buttner, John Innes Centre

Table 2.2. Plasmids and cloning vectors used in this work

Plasmid/ vectors	Selective marker	Relevant characteristics	Reference/Source
***E. coli* plasmids & phagemid**			
pAU227	Ampicillin	It has the entire helicase gene from the *SacI* site 215 nt upstream of the putative GTG start codon to the *NcoI* site 439 nt downstream of the stop codon cloned into the pUC120 polylinker	Stoehr 2001
pMAL-c2X	Ampicillin	Originated from pBR322. Used to express Maltose Binding Protein (MBP)-fusion proteins	New England Biolabs
pAU330	Ampicillin	Derived from pMAL-c2X. Used for the expression of the MBP-tagged ATG-version of SCO3550	Gislason 2005
pAU339	Ampicillin	Derived from pMAL-c2X. Used for the expression of the MBP-tagged GTG-version of SCO3550	Current study
pET15b	Ampicillin	Originated from pBR322. Used to express His-tagged proteins under control of the T7 promoter	Novagen
pAU338	Ampicillin	Derived from pET15b. Used for the expression of the His-tagged ATG-version of SCO3550	Current study

Table 2.2 continued: Plasmids and cloning vectors used in this work

Plasmid/ vectors	Selective marker	Relevant characteristics	Reference/Source
***E. coli* plasmids & phagemid**			
pGEM3	Ampicillin	Contains both the SP6 and T7 promoters. Used to make 5'-tailed artificial RNA substrates for assaying helicase activity	Promega. Gift from Dr Owtthrim, University of Alberta
pGEM3CS-	Ampicillin	Derived from pGEM3. Used to make 3'-tailed artificial RNA substrates for assaying helicase activity	Scheffner et al. 1989, Gift from Dr. Owtthrim
pGEM-MO1/2	Ampicillin	Derived from pGEM3. Used to make 5'-tailed artificial RNA substrate with 14 bp duplex region for assaying helicase activity	Scheffner et al. 1989, Gift from Dr. Owtthrim
Shuttle plasmids			
pIJ6902	Apramycin	Derived from pSET152. It contains thiostrepton -inducible promoter *ptipA* from *Streptomyces*	Gift from M. Buttner, John Innes Centre
pAU331	Apramycin	Derived from pIJ6902. It has the *sco3550* coding region from the predicted ATG start codon to 463 nt downstream of the stop codon	Gislason 2005
pAU336	Apramycin	Derived from pAU331. Used to express the His-tagged ATG-version of SCO3550 in *S. coelicolor*	Gislason 2005

selection or maintenance, cultures were grown in the presence of 50μg/mL Apramycin and/or 30 μg/mL thiostrepton. Strains were maintained as 20% glycerol spore stocks at -20^{0}C or as solid plate cultures at 4^{0}C.

2.1.6. Preparation of *S. coelicolor* glycerol stocks

Spore stocks were prepared as described by Hopwood et al. (1985).

2.2. DNA isolation and transformation

2.2.1. Preparation of *E. coli* chemically competent cells

Five milliliters of LB broth was inoculated with an isolated colony from a plate and grown at 37^{0}C on a rotating rack overnight. An aliquot of 250 μL was then used to inoculate 25 mL of LB medium and the culture was grown under the same conditions as above to an OD_{600} of approximately 0.6. The cells were harvested by centrifugation at 3000 rpm for 5 min, washed with 5 mL of 100 mM $MgCl_2$ and kept on ice for 5 min. The cells were then pelleted by centrifugation as above, washed with 5 mL of 100 mM $CaCl_2$ and kept on ice for 5 min before a further centrifugation. The final cell pellet was suspended in 100 mM $CaCl_2$ and 20% glycerol to a total volume of 1 mL. Aliquots of 50 μL were then flash frozen in dry ice and stored at -86^{0}C.

2.2.2. Transformation of *E. coli*

Purchased *E coli* DH5α chemically competent cells (Invitrogen) were transformed based on the manufacturer's recommendations. Other chemically competent cells used in this work (see above, Section 2.2.1) were transformed the same way as the purchased DH5α cells except that heat shocking was done at 42^{0}C for 45 seconds.

2.2.3. Isolation of plasmid DNA from *E. coli*

Plasmid DNA was isolated from *E. coli* using the alkali lysis method described by Sambrook et al. (1989).

2.3. DNA purification and analysis

2.3.1. Restriction digestion and cloning of DNA

Restriction endonuclease digestions of plasmids and PCR products were done according to the manufacturer's instructions (Roche, New England Biolabs or Fermentas). If required, 5'- or 3'- protruding termini of a DNA fragment were blunted with T4 DNA polymerase following the manufacturer's recommendation (Fermentas). Ligations were done using a vector to insert ratio of 1:3 for sticky end ligations and 1:5 for blunt-sticky end ligations. Ligation reactions contained 1X ligation buffer (50 mM Tris-HCl, pH-7.6, 10 mM $MgCl_2$, 5% PEG 8000 and 1 mM DTT), 1 mM ATP, 1 unit of T4 DNA ligase and the appropriate amount of the insert and vector fragments in a final volume of 20 µL. The blunt-sticky end ligations were done at 18^0C overnight while the sticky end ligations were done at 15^0C overnight.

2.3.2. Polymerase chain reaction (PCR)

Polymerase chain reaction was used to amplify DNA fragments for cloning. It was also used to screen transformants for the presence of insert DNA fragments. The standard PCR reaction contained 4 pmol of each primer, 0.2 mM of the dNTPs, 2% DMSO, 1X buffer 2 (Roche), 10-60 ng of plasmid DNA and 0.5 U of the appropriate polymerase in a final volume of 20 µL. Reactions for the amplification of DNA fragments for cloning were done with the same concentration of reactants, but with EXPAND™ High-Fidelity polymerase (Roche) in a total volume of 100 µL. PCR

reactions were done in a Biometra® T personal PCR machine (Montreal Biotech Inc.). The list of primers used in this study is shown in Table 2.3.

2.3.3. Agarose gel electrophoresis of DNA

DNA fragments over 1 kb were electrophoresed at 100 V on 1X TBE (Sambrook et al. 1989), 1% agarose gels. *Pst* I digested λ-DNA was used as the DNA molecular weight marker. Fragments smaller than 1 kb were separated on 1X TBE, 2% agarose gel with Molecular Weight Marker XIV (Roche) as the size standard. To facilitate loading of samples into the wells, and to monitor migration of the samples on gels, 1/4 volume of DNA loading dye containing 0.25% bromophenol blue and 40% sucrose was added to the samples. After electrophoresis, DNA bands in the gels were stained in ethidium bromide solution and the DNA bands were visualized by exposure to UV light.

2.3.4. Purification of DNA from agarose gels

DNA fragments were typically purified using the QIAquick DNA purification system (Qiagen). In some cases, electroelution into troughs described in Zhen and Swank (1993) and Maniatis et al. (1982) was used to obtain a higher yield of the purified DNA.

2.3.5. Polyacrylamide gel electrophoresis of DNA

DNA fragments lower than 1 kb were electrophoresed on 5% polyacrylamide gels with ratio of 29:1 acrylamide to the N, N'-methylene bisacrylamide. Electrophoresis was done in 1 X TBE at 200 V. Molecular weight markers III or XIV from Roche were used depending on the size of the fragments to be visualized. Preparation of the samples for loading and visualization were done as described in section 2.3.3.

Table 2.3. Oligonucleotide primers used

Primer	Sequence (5'-3')	Region of homology[+]	Use
BKL82	cgcgtctagacgacgtacttcagtgcc	+ 673 to +656	PCR for pAU339 construction
BKL87	gtgccggtggcgacgac	+341 to +325	PCR confirmation of transformants, sequencing
JST12-2	ggccatatgctcctggaccggctcgc	+118 to +137	PCR confirmation of transformants
KGI1	tctttcaccgaccggcactg	+488 to +469	PCR confirmation of transformants
KGI7	gggatcgatcgggtcgg	+85 to +69	Sequencing
KGI11	acaaggatccgctgcacaag	+3059 to +3073	PCR confirmation of transformants, sequencing
KGI12	tccttagtcatgttggtgcgctgcgtgctc	+4004 to +3285	PCR confirmation of transformants
WAZ4	cacgacgccttggtcc	+4 to +19	PCR for pAU339 construction

[+]Region of homology is measured from the predicted GTG translational start site of *sco3550*.

2.3.6. DNA sequencing

Automated sequencing was done using the Amersham ET Kit. The reaction included 20% DMSO, 5 ρmol of the sequencing primer, 8 μL of the ET mix and 100-500 ng double-stranded plasmid template in a total volume of 20 μL. The sequencing reactions were run with a regular protocol that required 25 cycles with temperature settings at 96^{0}C for 30 sec, 50^{0}C for 15 sec and 60^{0}C for 1 min, or with a high GC protocol of 30 cycles with temperature settings at 96^{0}C for 30 sec and 60^{0}C for 2 min. The 20 μL sequencing reactions were mixed with 2 μL NaOAC/EDTA in 1.5 mL eppendorf tube by pipetting up and down. Eighty microliters of 95% ETOH were then added and the suspensions were left on ice for exactly15 min. After microcentrifugation (min at rpm) the pellets were washed with 70% ETOH, air dried and stored at -20^{0}C until delivery to the Molecular Biology Service Unit (Dept of Biological Sciences) for automated sequencing.

2.4. Protein isolation, purification and analysis

2.4.1. Over expression and purification of recombinant SCO3550

E. coli expression hosts carrying plasmids for the expression of the recombinant proteins were grown at 37^{0}C to an OD_{600} around 0.6. Protein induction was done at room temperature for 4-6 hrs by adding isopropyl-β-D thiogalactoside (IPTG) to a final concentration of 0.4 mM. For the expression of His-tagged SCO3550 in *S. coelicolor*, cells were grown for 12-16 hrs prior to induction for 3.5 hrs with 30 μg/mL thiostrepton. Cell extracts were prepared as described below and affinity chromatography was used to purify the recombinant proteins. Recombinant 6xHis-tagged SCO3550 was purified using 500 μL Nickel-Nitriloacetic acid (Ni-NTA) matrix (Qiagen) and the instructions

outlined in Novagen Ni-NTA Purification Kit manual while 150 µL amylose resin was used to purify maltose binding protein (MBP)-tagged SCO3550 following the manufacturer's instructions (New England Biolabs). Proteins were purified under native conditions.

2.4.2. Preparation of *S. coelicolor* and *E. coli* cell extracts

Cell pellets of *S. coelicolor* were suspended in 1 X PBS containing 1 X complete, EDTA-free protease inhibitor cocktail (Roche). *E. coli* cell pellets were suspended in protein purification binding buffer (pH 8) containing 10 mM imidazole, 300 mM NaCl and 50 mM sodium phosphate buffer (Novagen nickel affinity chromatography) or in column buffer containing 20 mM Tris-HCl, pH 7.4, 200 mM NaCl and 1 mM EDTA (New England Biolabs amylose resin chromatography) in the presence of 1X protease inhibitor cocktail (Roche). Cells were lysed using Branson Sonifier 450 set at level 1 with a microprobe. The sonication was done 5 times for 15 sec on ice. Each 15 sec interval of sonication was followed by 15 sec of cooling. After sonication, cell lysates were centrifuged at 13000 rpm in a benchtop microcentrifuge for 10-30 min. Supernatants were used immediately for protein purification.

2.4.3. Quantification of protein

Total protein in cell free extracts and preparations of purified recombinant proteins was quantified using the procedure described in Bio-Rad Protein Assay Kit. Bovine gamma globulin was used as a standard.

2.4.4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Cell free extracts and purified fusion proteins were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Sambrook et

al. (1985). Up to 100 μg amount of proteins in cell free extracts and 3 μg of the recombinant proteins were loaded on mini gels (Bio-Rad Mini-PROTEAN® 3 system). Samples were prepared by adding 1/4 volumes of 2X SDS loading buffer and heat denaturation at 95^0C for 7 min prior to loading. Samples were electrophoresed on 6 or 7.5% polyacrylamide (29:1 Acrylamide: N, N'-Methylenbisacrylamide) resolving gels (1.5 mM Tris-HCl, pH 8:8 and 0.1% SDS) using stacking gels containing 3.2 % polyacrylamide, 0.5M Tris-HCl, pH 6.7 and 0.07% SDS. Pre-stained protein standards (Fermentas) were used as a molecular weight marker. Electrophoresis was done at 200 V in a Tris-glycine-SDS buffer (0.05 M Tris, 0.38 M glycine and 0.1 % SDS). After electrophoresis the gels were stained with Coomassie Brilliant Blue (Bio-Rad).

2.5. Enzyme assays

2.5.1. Nucleoside triphosphatase (NTPase) assay

NTPase activity of MBP-SCO3550 was monitored using a coupled spectrophotometric method as described by Iost et al., (1999) with some slight modifications. In this assay, NTP hydrolysis was linked to the oxidation of NADH using pyruvate kinase and lactate dehydrogenase resulting in the decrease in absorbance of NADH at 338 nm. Unless otherwise stated, assays were done in a reaction volume of 0.2 mL containing 20 mM HEPES buffer, pH 7.5, 50 mM KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 3 mM ATP, 0.3 mM NADH, 2 mM phosphoenolpyruvate, 0.6 units of pyruvate kinase, 0.74 units of lactate dehydrogenase, 20 μg of RNA, and 800 ng (approximately 0.03 μM) of purified MBP-SCO3550. The reactions were performed in triplicate in 96-well microtiter plates. The change in absorbance at 338 nm was

continuously measured for 30 min using a SpectraMAX Plus Spectrophotometer. Since the steady-state rate of NTP hydrolysis is equal to the steady-state rate of NADH oxidation, the rate of NTP hydrolysis was calculated using the extinction coefficient of NADH (6300 $M^{-1}cm^{-1}$).

2.5.2. RNA helicase assay

The RNA helicase assay reaction conditions were similar to those of Chamot et al., (2005). Unless otherwise stated, the total reaction volume was 20 µL and the reactions contained 20 mM HEPES-KOH, pH 7.5, 5mM $MgCl_2$, 3 mM ATP, 1 mM dithiothreitol (Promega), 200 µg/mL BSA (Roche), 24µg RNAseOUT (Invitrogen), 2000 cpm dsRNA (approximately 0.5 ρmol) and 1000 ng (0.4 µM) of the recombinant protein. The reaction was incubated at 37^0C for 30 min and quenched by the addition of 5 µL 2X SDS sample buffer (0.1M Tris-HCl, pH 6.8, 20% glycerol, 2% β-mercaptoethanol, 4% SDS, and 0.2% Bromophenol blue). Aliquots (15 µL) of each reaction were loaded onto 11% SDS-polyacrylamide (29:1 Acrylamide: N, N'-Methylenbisacrylamide) gels and electrophoresed at 200 V for 30 min. The gels were dried at 80^0C using a BioRad gel dryer with a Savant pump for 25 min and then observed after scanning with a Fluorescent Image Analyzer-5000 (FUJIFILM Medical Systems) . The intensity of the bands was quantified using ImageGauge V4.22 software (FUJIFILM Medical Systems). Unless otherwise stated, the measured intensity of the bands includes self-dissociation of the ds RNA substrates. The results of the experiments were only compared qualitatively since the reaction incubation time and/or amount of protein varied with each trial.

2.5.3. in vitro RNA transcription assays

Plasmids used to prepare DNA templates for the transcription assays have SP6 and T7 RNA polymerase promoter sequences. The plasmids were linearized using a suitable restriction endonuclease and aliquots of the samples were run on 1% TBE agarose gels to check for complete digestion. As a precaution against extraneous transcript production from DNA templates with 3' overhangs (Schenborn and Mierendorf 1985), *Hae* II-restricted plasmid was blunted with T4 DNA polymerase (Fermentas). Linearized plasmids were then extracted with phenol:chloroform:isoamyl alcohol followed by chloroform extraction. They were then precipitated with a 2.5X volume of ethanol and 1/10 volume of 3 M sodium acetate and redissolved in TE buffer to a final concentration of 1μg/mL. The transcription assays were done using Riboprobe in vitro Transcription System (Promega) following the manufacturer's recommendations. For radiolabeled transcripts, the transcription reaction contained 1X Transcription Optimized Buffer, 10 mM DTT, 20 U of Recombinant RNasin Ribonuclease Inhibitor (Promega), 0.125 mM of rATP, rCTP and rGTP, 12 μM of rUTP (if the radiolabeled nucleotide was uridine 5'- triphosphate) or 12 μM of rGTP (if the radiolabeled nucleotide was guanosine 5'- triphosphate), 1-2 μg of template DNA, 50 μCi of α-^{32}P-rUTP or α-^{32}P-rGTP and 20 U of the appropriate RNA polymerase in a reaction volume of 20 μL. Components were added in the order listed. The mixture was kept at room temperature while each successive component was added to avoid precipitation of DNA at 4^0C in the presence of spermidine, which is a component of the Transcription Optimized Buffer. The reaction for the non-radiolabeled transcripts was scaled up 3-5X. The reactions were incubated at 37-40^0C for 1-2 hrs and the transcripts purified as described below.

2.5.4. Preparation of RNA helicase substrates

The structures of the substrates used are shown in Fig.3.6. The 5'-tailed substrate (RNA II) shown in Fig. 3.6A was prepared by annealing transcripts obtained after transcribing from the SP6 promoter on *Bam* HI-digested pGEM3 as template (radioactive-transcript) and from T7 promoter using *Hind* II- digested pGEMMO1/2 as template (non-radioactive-transcript) (Pause *et al.*, 1993). The transcription assays were done using the Riboprobe in vitro Transcription System (Promega) as described above. The radiolabeled transcript was prepared using either α-^{32}P- GTP or α-^{32}P- UTP (PerkinElmer) as the radioactive nucleotide. The single-stranded transcripts were resolved on a 10% polyacrylamide (29: 1 Acrylamide: N, N'-Methylenbisacrylamide) gel containing 2.7 M urea in 1X TBE (89mM Tris base, 89mM Boric acid, 2mM EDTA). The non-radioactive transcript was run along with a radiolabeled guide to help locate its position on the gel. The label bands were visualized on X-ray film placed on top of the gel wrapped in saran. Strips of the gel containing the transcripts were cut out and chopped finely with scalpel. Transcripts were then eluted with shaking overnight in a solution containing 0.5 M ammonium acetate, pH 7, 0.1% SDS and 10 mM EDTA. After pelleting the polyacrylamide gel pieces, the supernatant was extracted using 1X phenol/chloroform followed by 1X chloroform and precipitated with 2.5X 95% ethanol. Both the radiolabeled and unlabeled transcripts were then suspended in 100 µL 1X annealing buffer (20 mM HEPES-KOH pH 7.6, 0.5 M NaCl, 1 mM EDTA, pH 8, and 0.1% SDS) and incubated at 95^{0}C for 5min followed by a slow cooling to 37^{0}C and incubation at 37^{0}C for 2hrs. The resulting dsRNA was purified by electrophoresis on a 6% native polyacrylamide (29: 1 Acrylamide: N, N'-Methylenbisacrylamide) gel in 1X

TBE and eluted from the gel as described above. The precipitated dsRNA was then redissolved in TE buffer at a concentration of 1000 cpm/uL and stored at -20^{0}C. The 5' dsRNA substrate shown in Fig. 3.6B was prepared as described above after transcription of *Acc* I-digested pGEM3 with T7 RNA polymerase and *Sac* I-digested pGEM3 with SP6 RNA polymerase with either strand radiolabeled (Flores-Rozas and Hurwitz, 1993). The 3' dsRNA substrate shown in Fig. 3.6C was prepared by digesting pGEM3CS$^-$ plasmid with *Rsa* I and transcribing with T7 RNA polymerase (103 nt non-radiolabeled transcript) and by digesting the pGEM3CS$^-$ plasmid with *Hae* II and transcribing with SP6 RNA polymerase to give 107 nt radiolabeled transcript (Scheffner et al., 1989). The preparation of the 3' dsRNA substrate shown in Fig. 3.6D was similar to that of the 3' dsRNA substrate in Fig. 3.6C except that the radiolabeled transcript (57 nt) was prepared from *Pvu* II digested pGEM3CS$^-$. The non-radiolabeled transcript was prepared by digesting pGEM3CS$^-$ plasmid with *Rsa* I and transcribing with T7 RNA polymerase. The purification of all ssRNA transcripts was done as described above. For the dsRNA substrates shown in Figs. 3.6B, C and D, the relevant ssRNA transcripts were annealed by incubation at 95^{0}C for 5 min followed by incubation at 65^{0}C for 30 min and slow cooling to room temperature for 12 hrs.

CHAPTER 3:

RESULTS

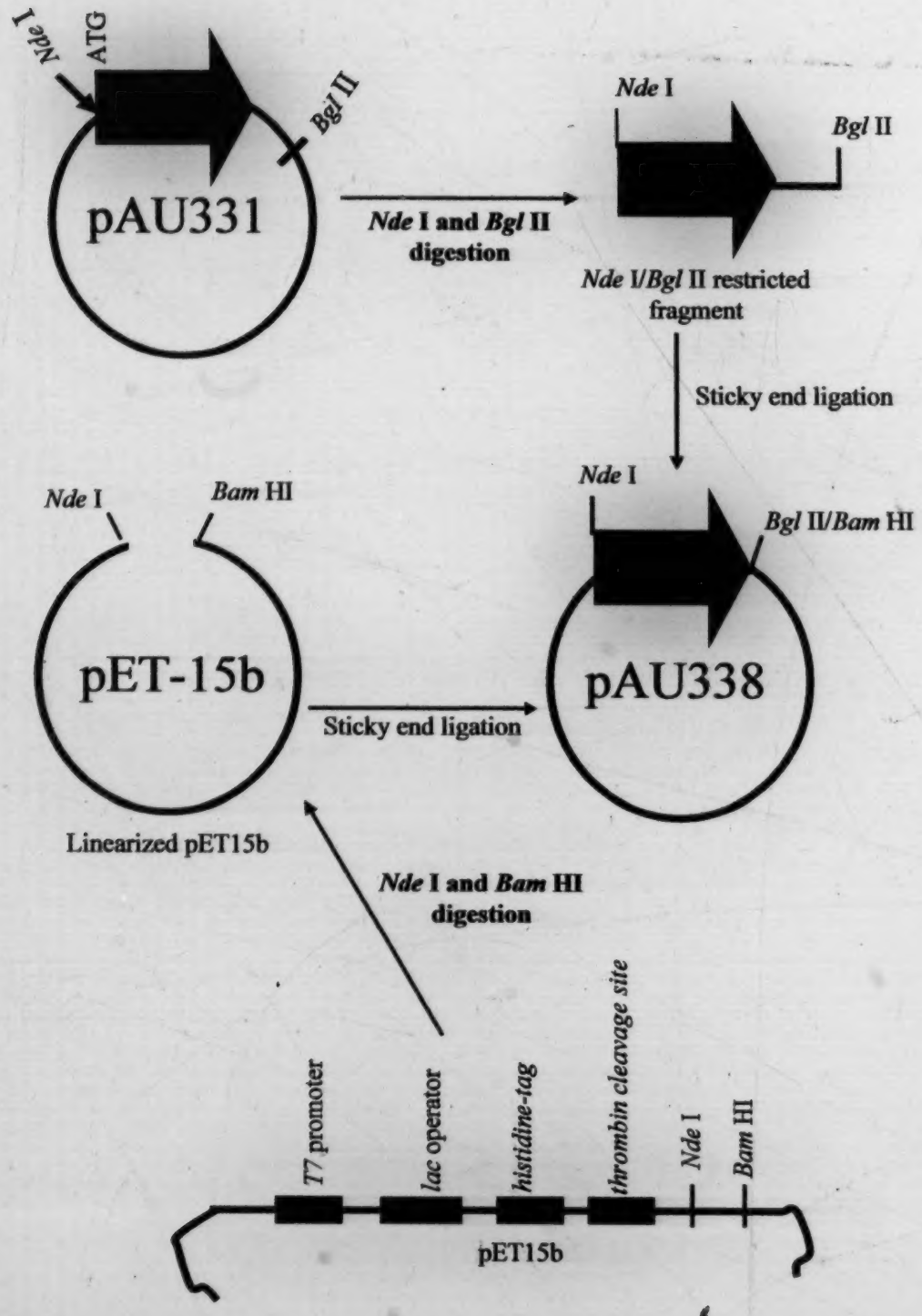
3.0. Results

3.1. Expression and purification of recombinant SCO3550

Previous in vitro assay of SCO3550 fused from its proposed ATG start codon to the maltose-binding protein tag has shown ATP-independent unwinding activity. Although there are a few examples of RNA helicases that exhibit ATP-independence, it is unusual for true RNA helicases. Since one possible reason for the observed ATP-independent unwinding activity of the MBP-ATGSCO3550 is that the relatively large maltose-binding protein tag might affect the enzymatic activity of the recombinant SCO3550 (Gislason 2005) (see section 1.6) a new fusion protein expression vector was constructed. To create this new vector, *ATGsco3550* was cloned into pET15b (Novagen) and expressed as a recombinant His-tagged protein. The His-tag consists of 6 histidine residues of an approximate molecular weight of 0.84 kDa, which is about 55 times smaller than the maltose-binding protein tag. The strategy used for cloning the *His_6-ATGsco3550* is shown in Fig 3.1. pET15b has a *lac* operator sequence downstream of a T7 promoter along with the natural promoter and coding sequence for the *lac* repressor gene divergently oriented from the T7 promoter. Also present is the sequence encoding a thrombin cleavage site sandwiched between the His-tag coding sequence and a multiple cloning site. The T7*lac* promoter system minimizes the basal expression of the target gene that might be detrimental to the expression host (Studier et al. 1990; Dubendorf and Studier 1991).

The recombinant His_6-ATGSCO3550 was expressed in *E. coli* strain BL21(DE3) that is deficient in *lon* protease and lacks the *ompT* outer membrane protease that can cause degradation of proteins during purification (Grodberg and Dunn 1988). The

Figure 3.1. Strategy for creation of a His_6-ATGSCO3550 overexpression vector. An *Nde* I/*Bgl* II fragment, starting from the ATG translational start codon to the *Bgl* II restriction site downstream of the *sco3550* stop codon, was isolated after restriction digestion of pAU331 (see Table 2.2). pET-15b (depicted as a straight line in the figure) was digested with *Nde* I and *Bam* HI to obtain a linearized plasmid. After purification, the fragments were ligated to give pAU338. The red arrow indicates the *sco3550* open reading frame.

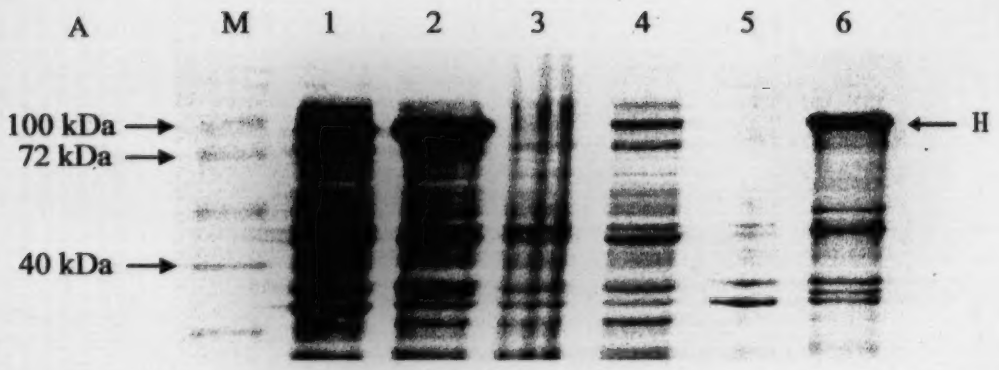

Nde I
ATG
Bgl II
pAU331
Nde I and Bgl II digestion
Nde I
Bgl II
Nde I/Bgl II restricted fragment
Sticky end ligation
Nde I
Bam HI
Nde I
Bgl II/Bam HI
pET-15b
Sticky end ligation
pAU338
Linearized pET15b
Nde I and Bam HI digestion
T7 promoter
lac operator
histidine-tag
thrombin cleavage site
Nde I
Bam HI
pET15b

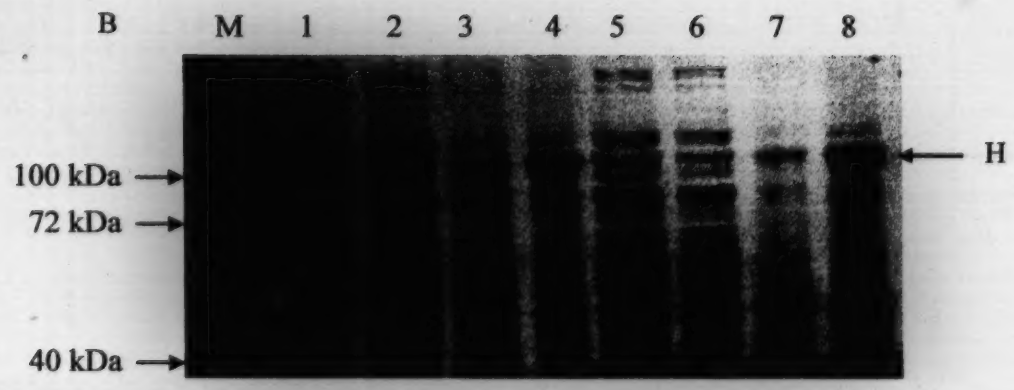
expression host is a lysogen of bacteriophage DE3, which carries a DNA fragment that codes for T7 RNA polymerase, whose expression is under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG) inducible *lacUV5* promoter (Studier and Moffatt 1986). Addition of IPTG to a growing culture of the expression host therefore induces T7 RNA polymerase production, which in turn transcribes the target gene encoded on the plasmid.

The initial expression of His_6-ATGSCO3550 based on the manufacturer's recommendations (Novagen) for growth and induction led to the aggregation of the protein in the insoluble cytoplasmic fraction (Fig.3.2A). Several attempts to improve on the solubility of the recombinant proteins were made. It is known that any factor that decreases the expression level of a recombinant protein reduces aggregation of the protein, thus increases its solubility. For the above reason, the recombinant His_6-ATGSCO3550 was expressed at lower temperatures (30^0C, room temperature and 17^0C) and in the presence of a reduced amount of IPTG. Additionally, the expression host was grown to stationary phase before the addition of IPTG inducer, since protein expression in older cells is further reduced compared to expression levels in exponentially growing cells.The experiments were done using the factors mentioned above, either singly or in combination. However, there was no significant improvement in the amount of the recombinant protein produced in the soluble cytoplasmic fraction (Fig. 3.2B).

Despite the expression of the recombinant His_6-ATGSCO3550 mainly as aggregates in the BL21(DE3) host, some proportion of the protein was expressed in the soluble form even in the uninduced control cells (Fig. 3.2B). It was therefore decided that the recombinant protein would be purified from cells grown to stationary phase without IPTG treatment. The protein was purified using affinity chromatography as described in

Figure 3.2. Expression of His_6-ATGSCO3550 in *E. coli.* (A) Initial expression of His_6-ATGSCO3550. The cells carrying the recombinant plasmid were grown at 30^0C to an OD_{600} of 0.7 before induction with IPTG (0.4 mM) and further incubation at 30^0C for 5 hrs. The final OD_{600} values were taken for the cultures and these values were used to determine the amount of the cell lysates to be loaded on a mini gel (Novagen). Uninduced cells grown under the same conditions served as a control (Lane 1). Lane 2 is the induced total cell protein, lane 3 is the uninduced soluble cytoplasmic fraction, lane 4 is the induced soluble cytoplasmic fraction, lane 5 is the uninduced insoluble cytoplasmic fraction, and lane 6 is the induced insoluble cytoplasmic fraction. In both panels A and B, lane M is the pre-stained protein standards while the H denotes the position of His_6-ATGSCO3550. The proteins were resolved on a 7.5% polyacrylamide gel and stained with Coomassie Brilliant Blue. (B) Optimization of the His_6-ATGSCO3550 expression. The cells were grown at room temperature to an OD_{600} of 2.92. After addition of IPTG (0.05-0.1 mM), they were grown a further for 3 hrs at 17^0C. Lane 1 is the uninduced soluble cytoplasmic fraction, lane 2 is the 0.05 mM IPTG induced soluble cytoplasmic fraction, lane 3 is the uninduced insoluble cytoplasmic fraction, lane 4 is the 0.05 mM IPTG induced insoluble cytoplasmic fraction, lane 5 is the uninduced soluble cytoplasmic fraction, lane 6 is the 0.1 mM IPTG induced soluble cytoplasmic fraction, lane 7 is the uninduced insoluble cytoplasmic fraction and lane 8 is the 0.1 mM IPTG induced insoluble cytoplasmic fraction. Samples were resolved and gels visualized as in (A).


A
M
1
2
3
4
5
6
100 kDa
72 kDa
40 kDa
H

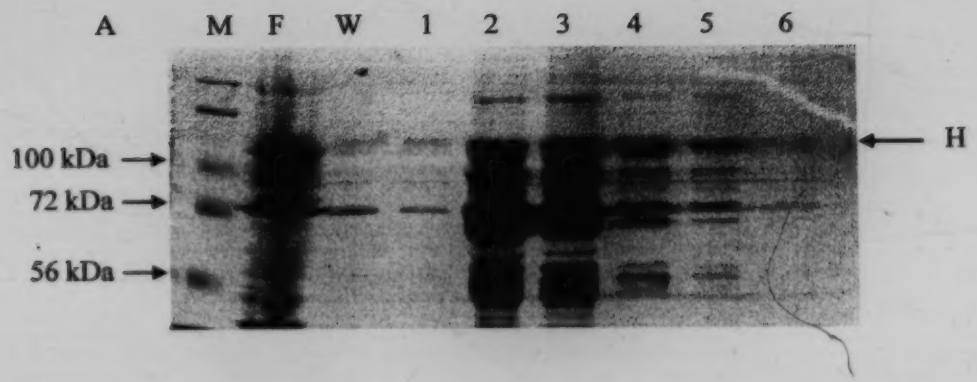

B
M
1
2
3
4
5
6
7
8
100 kDa
72 kDa
40 kDa
H

Materials and Methods (section 2.4.1). There was degradation and what seemed to be non-specific binding of unwanted proteins after the purification (Fig.3.3A), and an increase in both the amount of protease inhibitor cocktail (Roche) and the stringency of washing did not improve the quality or purity of the purified protein. Using similar purification conditions, Kent Gislason was able to purify the His_6-ATGSCO3550 expressed in *S. coelicolor* from the recombinant plasmid pAU336 (see Table 2.2.) (Gislason 2005). Therefore, the His_6-ATGSCO3550 used for the RNA unwinding experiments described below was the recombinant protein expressed and purified from *S. coelicolor* (Fig. 3.3B). This was considered to be a better option since the native *S. coelicolor* host would permit any necessary post-translational modifications of the protein. Lack of post-translational modification of the MBP-ATGSCO3550 purified from *E. coli* might also contribute to its ATP-independent unwinding activity observed previously. It is worth mentioning that the initial expression of the recombinant His_6-ATGSCO3550 was done in *E. coli* instead of *S. coelicolor* because the latter is a poor expression host, and it was thought that enough protein might not be produced for the biochemical assays.

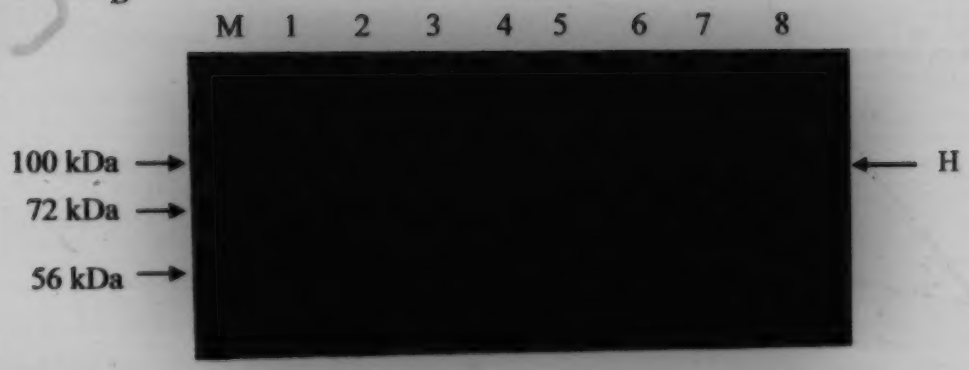
The second concern from the previous study (Gislason 2005) was that the MBP-ATGSCO3550 might not be the fully active form of the protein. The possibility existed that absence of the 39 amino acid residues between the predicted GTG and ATG translational start codons might alter the biochemical activity of the protein (see section 1.6). This was a larger concern than the possible steric effects of the maltose-binding protein fusion on SCO3550 activity because even though the large maltose-binding

Figure 3.3. Purification of recombinant His_6-ATGSCO3550. (A & B) Affinity purification of recombinant His_6-ATGSCO3550 expressed in *E. coli* and *S. coelicolor*, respectively. Lane M is the pre-stained protein standards (Fermentas), lane F is the flow-through from the Ni-NTA column, lane W is the wash from the Ni-NTA column (40 mM imidazole) and the lanes labeled with the numerals are the fractions containing the His_6-ATGSCO3550 after elution with 250 mM imidazole. The flow through and wash fraction are not shown in panel B. The proteins were resolved on a 7.5% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. The H in panels A and B denotes the position of the His_6-ATGSCO3550 on the gels.

A


M F W 1 2 3 4 5 6
100 kDa
72 kDa
56 kDa
H

B


M 1 2 3 4 5 6 7 8
100 kDa
72 kDa
56 kDa
H

protein tag might influence the enzymatic activities of proteins to which it is fused, it has been shown to enhance the solubility of proteins expressed in *E. coli*, better than GST- and Trx-based systems (Kapust and Waugh 1999). In most cases, a maltose-binding fusion protein is soluble and fully active. *sco3550* was therefore cloned into pMAL-c2X (New England Biolabs) and expressed as a recombinant maltose-binding protein-tagged GTGSCO3550 in order to compare its biochemical activities to those of the ATG-version of the protein. Constructs were made for the two putative isoforms by cloning the entire open reading frames coding for the putative ATGSCO3550 and GTGSCO3550 to give pAU330 (Gislason 2005) and pAU339 (this study), respectively. The strategy used for cloning pAU339 was the same as previously used to construct the ATGSCO3550 fusion vector pAU330, and is shown in Fig. 3.4. The recombinant proteins were expressed under the control of the pMAL-c2X-encoded, strong "ptac" promoter in *E. coli* strain BL21(DE3) and purified to homogeneity using affinity chromatography on an amylose resin column following the manufacturer's instructions (New England Biolabs). The purity and the expected size of the recombinant proteins were verified by polyacrylamide gel electrophoresis (Fig 3.5).

3.2. RNA unwinding activity of SCO3550

As the first step to examine the enzymatic activities of SCO3550, the His-tagged ATG-version of SCO3550 (His_6-ATGSCO3550), which was purified from *S. coelicolor*, was tested for RNA duplex destabilization. In this assay, duplex unwinding leads to the accumulation of radiolabeled single-stranded RNA molecules that can be observed after scanning (in this case a Fluorescent Image Analyzer-5000, FUJIFILM Medical Systems, was used). The parameters for the unwinding reactions are described in section 2.5.2 and

Figure 3.4. Strategy used in the construction of pAU339. The entire coding region of the *GTGsco3550* starting from the CAC codon immediately downstream of the predicted GTG-start codon to 439 nt downstream of the TAG stop codon of *sco3550* was cloned into the pMAL-c2X expression vector. The first step involved cloning of the 5' *GTGsco3550* 564 bp PCR product in-frame to *malE,* which expresses maltose-binding protein (MBP). The required region was amplified with WAZ4 (forward primer) and BKL82 (reverse primer) with pAU227 as the template to obtain a 680 bp product. After removal of the adenylate homopolymers from the PCR product with T4 DNA polymerase, the enzyme was heat-inactivated and the PCR product digested with *Bam* HI resulting in a 564 bp product. The 564 bp product was then cloned into the *Xmn* I/*Bam* HI linearized pMAL-c2X to obtain pMAL-c2X-5'-*GTGsco3550.* The product was sequenced to ensure sequence integrity. The pMAL-c2X-5'-*GTGsco3550* was then digested with *Hind* III and treated with T4 DNA polymerase to generate a linearized blunt-end product. The T4 DNA polymerase was heat-inactivated and the pMAL-c2X-5'-*GTGsco3550* linearized product was further digested with *Bam* HI. This product was used in the final step of the cloning. The second step involved preparation of the 3' *sco3550* from pAU227. The plasmid was digested with *Nco* I and the resulting sticky end removed by treatment with T4 DNA polymerase. The T4 DNA polymerase was heat-inactivated and the linearized plasmid digested with *Bam* HI to obtain a 2319 bp 3' *sco3550* product. The final step involved cloning of the 3' *sco3550* product into the linearized pMAL-c2X-5'-*GTGsco3550* to obtain pAU339. Fragment sizes are not to scale.

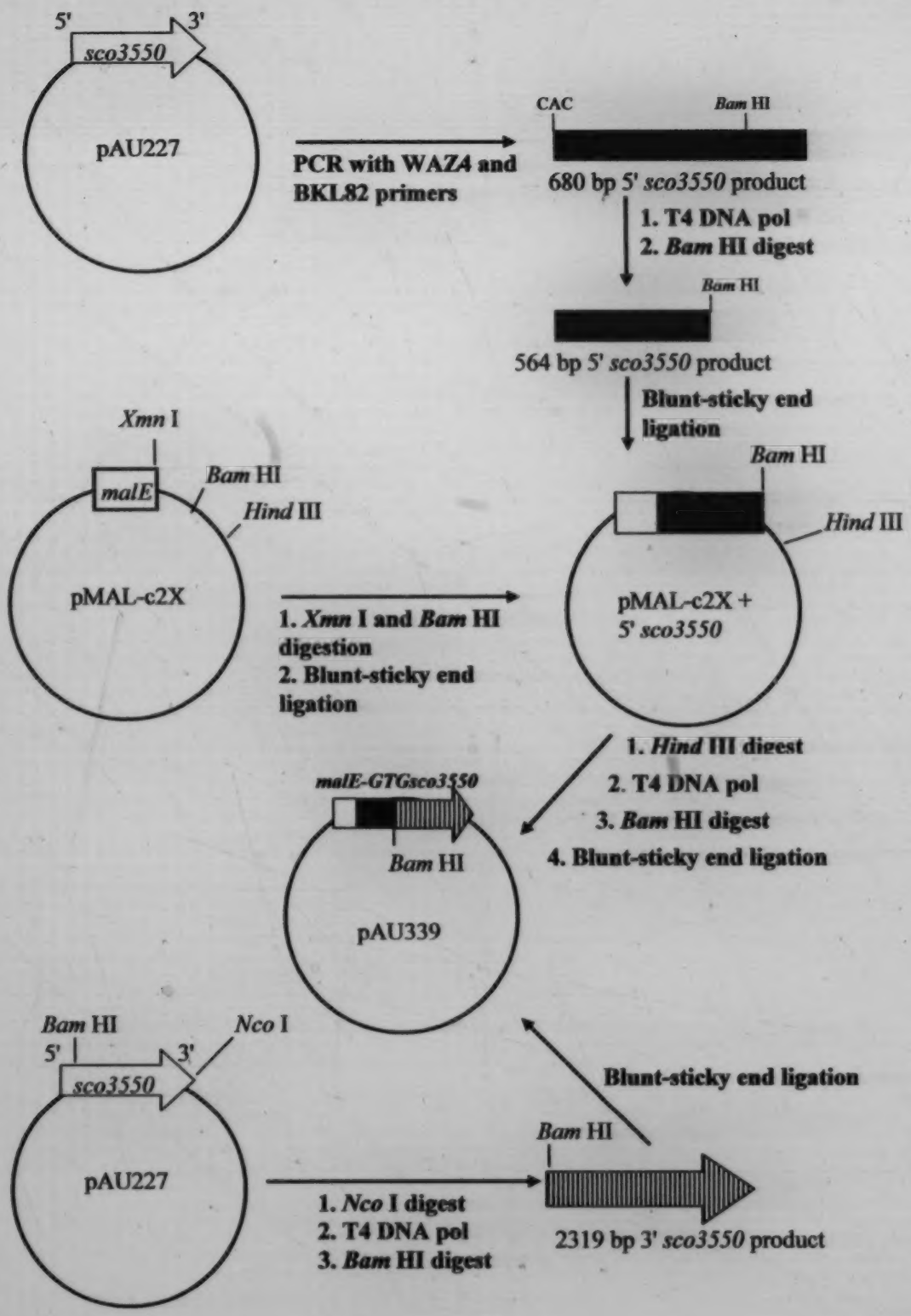
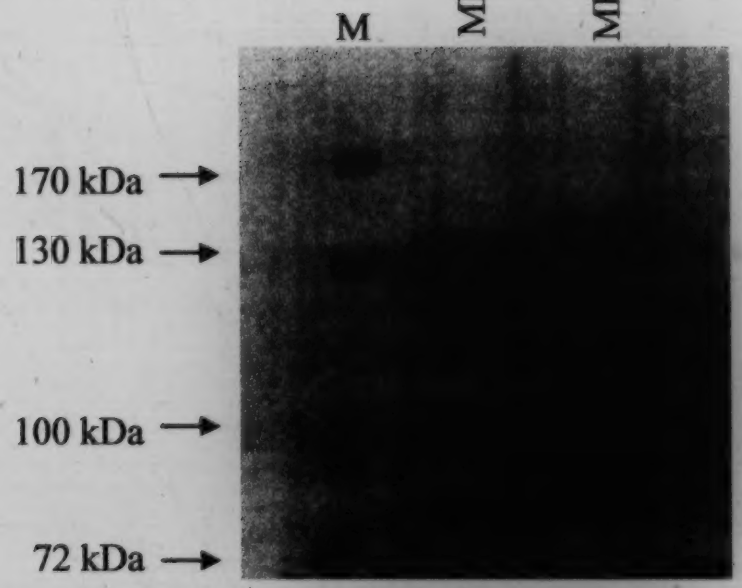

5'
3'
sco3550
pAU227
PCR with WAZA4 and BKL82 primers
CAC
Bam HI
680 bp 5' sco3550 product
1. T4 DNA pol
2. Bam HI digest
Bam HI
564 bp 5' sco3550 product
Blunt-sticky end ligation
Xmn I
malE
Bam HI
Hind III
pMAL-c2X
1. Xmn I and Bam HI digestion
2. Blunt-sticky end ligation
Bam HI
Hind III
pMAL-c2X + 5' sco3550
1. Hind III digest
2. T4 DNA pol
3. Bam HI digest
4. Blunt-sticky end ligation
malE-GTGsco3550
Bam HI
pAU339
Bam HI
Nco I
5'
3'
sco3550
pAU227
1. Nco I digest
2. T4 DNA pol
3. Bam HI digest
Blunt-sticky end ligation
Bam HI
2319 bp 3' sco3550 product

Figure 3.5. Affinity purification of recombinant MBP-SCO3550. The putative SCO3550 isoforms fused to maltose-binding protein (MBP) were overproduced in *E. coli* and purified by affinity chromatography on an amylose resin column. Lane M is the pre-stained protein standards (Fermentas). The proteins were resolved on a 6% polyacrylamide gel and stained with Coomassie Brilliant Blue.

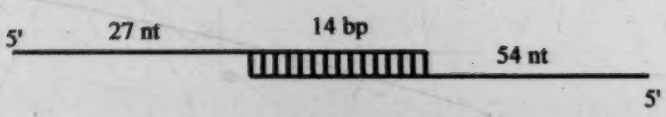

M
MBP-ATGSCO3550
MBP-GTGSCO3550
170 kDa
130 kDa
100 kDa
72 kDa

were done using the standard 5' dsRNA substrate shown in Fig.3.6A. As a measure against self-dissociation of the dsRNA substrate, an aliquot of the substrate was incubated at 37^0C in the absence of the test protein along with other test samples. The substrate treated at 95^0C served as the positive control for duplex destabilization. After 30 min of incubation of the substrate with up to 1400 ng amount of the His_6-ATGSCO3550 purified from *S. coelicolor*, no unwinding was observed (Fig. 3.7). The experiment was repeated two more times, but unwinding of the artificial dsRNA substrate was never observed. Since the failure to observe unwinding could have resulted from an inactive protein preparation that was due to its storage at -86^0C prior to its use in the assay, the assays were repeated with freshly purified protein using the conditions described above. Again, no unwinding was observed after two attempts. It was concluded that the His_6-ATGSCO3550 might be expressed in *S. coelicolor* in an inactive form or that the tag might cause non-functional folding of the protein.

The dsRNA unwinding activities of SCO3550 were then tested with both the ATG and GTG-versions of SCO3550 fused to the maltose-binding protein tag using similar unwinding reaction conditions and the 14bp duplex region RNA substrate (Fig. 3.6A) as described for the His_6-ATGSCO3550. As observed in the previous study (Gislason 2005), an increase in the amount of the recombinant proteins (Fig. 3.8, A&B), which had been purified from *E. coli,* or the incubation time (3.9, A&C) led to an increase in the amount of the ssRNA generated from the dsRNA substrates. Since binding of proteins that lack unwinding activity to the artificial dsRNA substrates could cause partial dissociation of the RNA duplex, it was advised that a substrate with a longer duplex region be used for the assays. The substrate shown in Fig. 3.6B with 26bp duplex

Figure 3.6. Structure of dsRNA substrates used for the RNA helicase assays. (A & B) 5'-tailed dsRNA substrates. (C &D) 3'-tailed dsRNA substrates. U. RNA is the upper strand RNA and L. RNA is the lower strand RNA. nt denotes nucleotides and bp stands for base pairs. The substrates were prepared as described in Materials and Methods (see sections 2.5.3 & 2.5.4) and were radiolabeled with α-^{32}P-GTP or α-^{32}P-UTP. The complementary sequences of the RNA substrates are underlined.

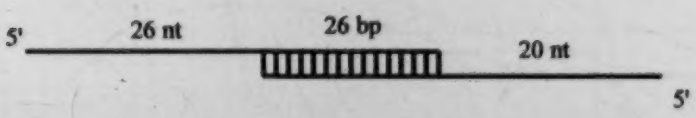
A



U. RNA 5' GAAUACAAGCUUGCAUGCCUGCAGGUCGACUCUAGAGGAUC 3'

L. RNA 5' GGGAGACCGGAAUUCCCCAUGGCUGACUAAUUUUUUUUAU
UUAUGCAGAGGGGGGAUCCUCUAGAGUC 3'

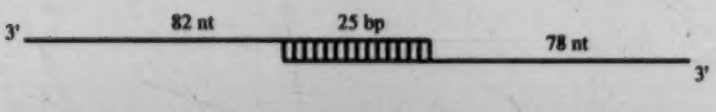
B



U. RNA 5' GAAUACAAGCUUGCAUGCCUGCAGGUCGACUCUAGAGGAU
CCCCGGGUACCG 3'

L. RNA 5' GGGAGACCGGAAUUCGAGCUCGGUACCCGGGGAUCCUCUA
GAGUCG 3'

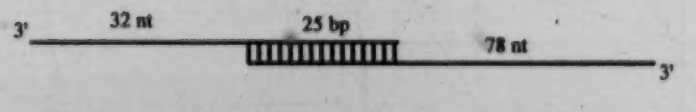
C



U. RNA 5' GAAUACAAGCUAAUUCCGGUCUCCCUAUAGUGAGUCGUAU
UAAUUUCGAUAAGCCAGCUGCAUUAAUGAAUCGGCCAACG
CGCGGGGAGAGGCGGUUUGCGUAUUGU 3'

L. RNA 5' GGGAGACCGGAAUUAGCUUGUAUUCUAUAGUGUCUCCUAA
AUCGUAUGUGUAUGAUACAUAAGGUUAUGUAUUAAUUGUA
GCCGCGUUCUAACGACAAUAUGU 3'

D



U. RNA 5' GAAUACAAGCUAAUUCCGGUCUCCCUAUAGUGAGUCGUAU
UAAUUUCGAUAAGCCAG 3'

L. RNA 5' GGGAGACCGGAAUUAGCUUGUAUUCUAUAGUGUCUCCUAA
AUCGUAUGUGUAUGAUACAUAAGGUUAUGUAUUAAUUGUA
GCCGCGUUCUAACGACAAUAUGU 3'

Figure 3.7. His_6-ATGSCO3550 purified from *S. coelicolor* does not unwind dsRNA. The assay tested 5' to 3' unwinding and was done as described in the Materials and Methods (see section 2.5.2). The experiments were done five times and representative results are shown. The lane labeled dsRNA is the control substrate incubated in the absence of protein at 37^0C, while the lane labeled ssRNA is the boiled control substrate.

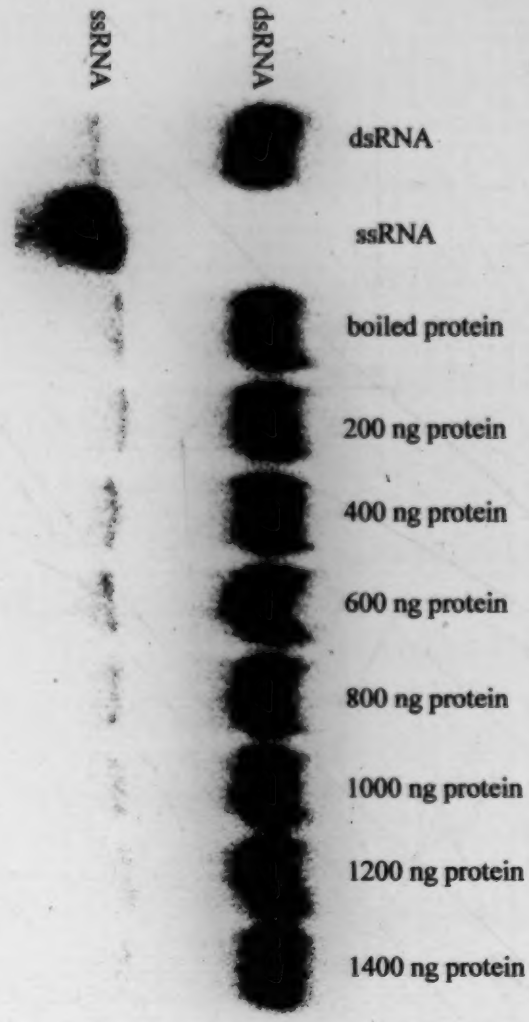
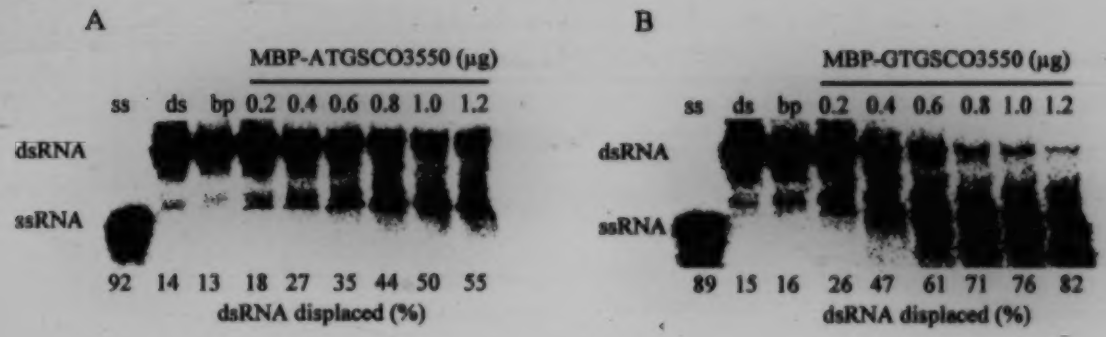
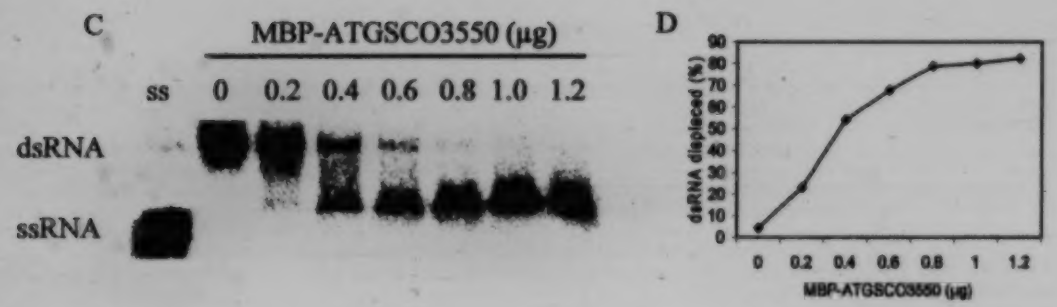

ssRNA
dsRNA
dsRNA
ssRNA
boiled protein
200 ng protein
400 ng protein
600 ng protein
800 ng protein
1000 ng protein
1200 ng protein
1400 ng protein

Figure 3.8. Influence of enzyme concentration on 5' to 3' unwinding by SCO3550. (A&B) The effect of increasing amounts of MBP-ATGSCO3550 and MBP-GTGSCO3550 on the 5' to 3' unwinding of artificial RNA substrate with a 14bp double-stranded region, respectively. The experiments were done four times using MBP-ATGSCO3550 and three times with the MBP-GTGSCO3550. Representative results are shown. (C&E) The effect of increasing amounts of MBP-ATGSCO3550 and MBP-GTGSCO3550 on the 5' to 3' unwinding of artificial RNA substrate with a 26bp double-stranded region, respectively. The experiments were done twice using MBP-GTGSCO3550 (representative result is shown) and once with the MBP-ATGSCO3550 (the result is shown). (D&F) Quantitation of the results in C and E, respectively. ss in all panels stands for the boiled control substrate, while ds and bp in panels A and B are the control substrate incubated in the absence of protein at 37^0C and the control reaction containing 1.2 μg heat-inactivated SCO3550, respectively.


A
MBP-ATGSCO3550 (µg)
ss ds bp 0.2 0.4 0.6 0.8 1.0 1.2
dsRNA
ssRNA
92 14 13 18 27 35 44 50 55
dsRNA displaced (%)
B
MBP-GTGSCO3550 (µg)
ss ds bp 0.2 0.4 0.6 0.8 1.0 1.2
dsRNA
ssRNA
89 15 16 26 47 61 71 76 82
dsRNA displaced (%)


C
MBP-ATGSCO3550 (µg)
ss 0 0.2 0.4 0.6 0.8 1.0 1.2
dsRNA
ssRNA
D
dsRNA displaced (%)
0 10 20 30 40 50 60 70 80 90
0 0.2 0.4 0.6 0.8 1 1.2
MBP-ATGSCO3550 (µg)

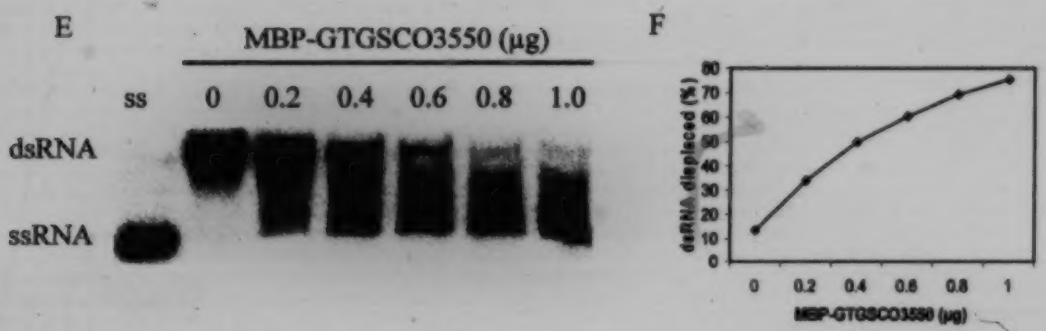
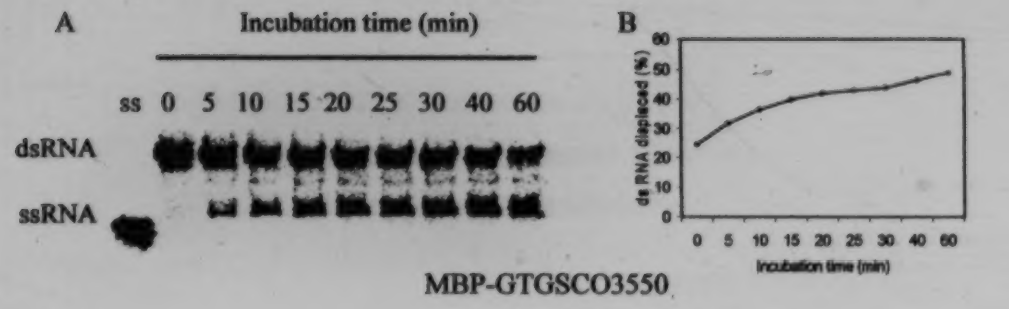
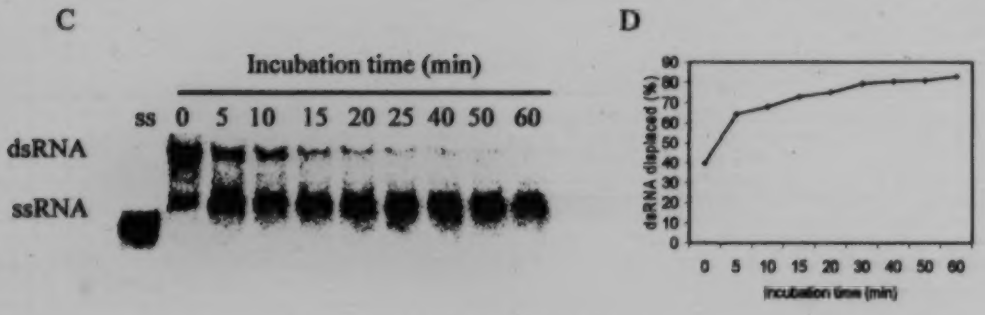

E
MBP-GTGSCO3550 (µg)
ss 0 0.2 0.4 0.6 0.8 1.0
dsRNA
ssRNA
F
dsRNA displaced (%)
0 10 20 30 40 50 60 70 80
0 0.2 0.4 0.6 0.8 1
MBP-GTGSCO3550 (µg)

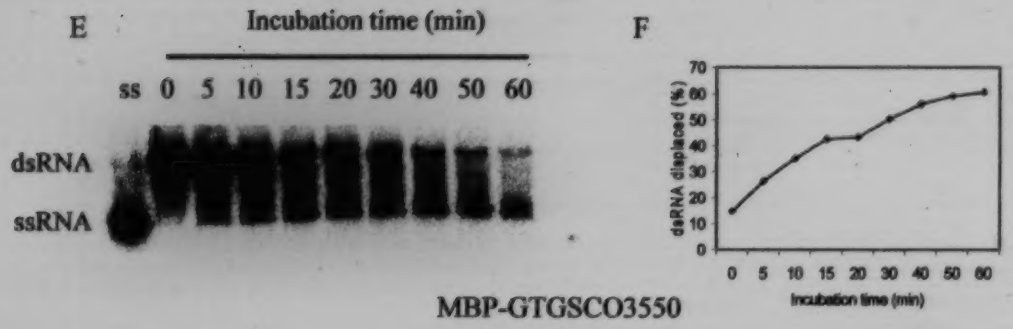
Figure 3.9. Effect of incubation time on the 5' to 3' unwinding activity of SCO3550. Incubation over time with the MBP-GTGSCO3550 (A & E) and MBP-ATGSCO3550 (C & G). The assays for A and C were done with1000 ng of the recombinant SCO3550 with the substrate described in Fig. 3.6A, while those of E and G were done with 300 ng of the recombinant SCO3550 using the substrate shown in Fig. 3.6B. The experiments with MBP-GTGSCO3550 were done once and the results are shown, while the experiments with MBP-ATGSCO3550 were done twice and representative results are shown. The right panels (B, D, F & H) are the quantification of the results shown in the left panels. The ss in the left panels denote the boiled substrate control (no protein) and the ds in panel G denotes double-stranded RNA substrate incubated in the absence of protein at 37^0C. The unwinding reactions were done as described in section 2.5.2.



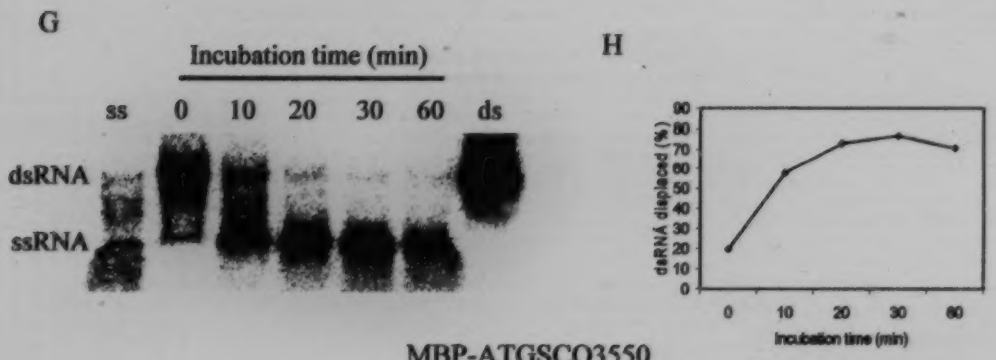
MBP-GTGSCO3550



MBP-ATGSCO3550



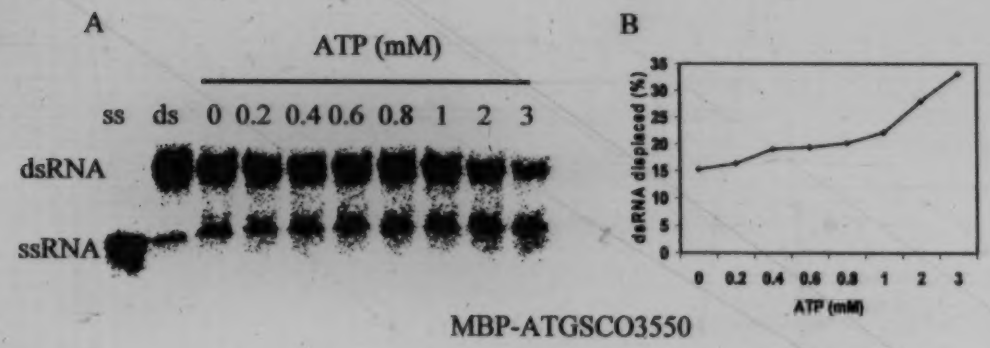
MBP-GTGSCO3550



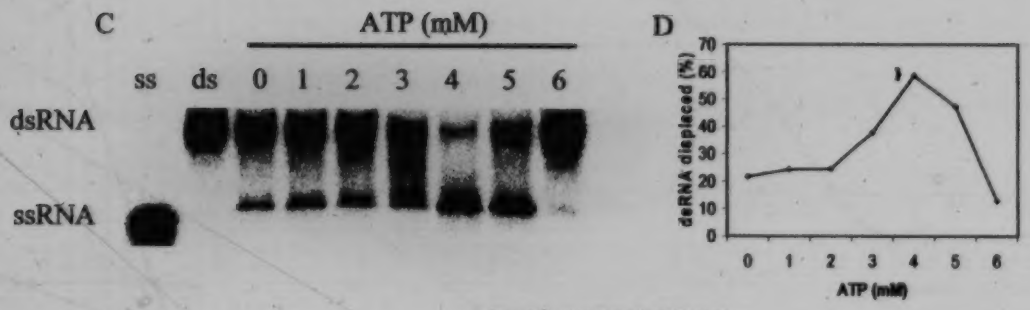
MBP-ATGSCO3550

region was therefore constructed and used for the assays described above. Again, an increase in the incubation time (Fig. 3.9, E&G) and the amount of the recombinant proteins (Fig. 3.8, C&E) led to an increase in the dsRNA substrate displaced. This indicates that duplex destabilization was not caused simply by binding of the recombinant proteins to the substrates. In contrast to the previous work done by Kent Gislason in which the MBP-ATGSCO3550 showed ATP-independent activity, the unwinding activities are energy-dependent because they increase with an increase in the amount of ATP (Fig. 3.10). In the presence of 4 mM ATP greater than 64% of the RNA duplex was unwound in the case of the MBP-ATGSCO3550 (Fig. 3.10C); while more than 70% unwinding was observed for the MBP-GTGSCO3550 at a 3 mM ATP concentration (Fig. 3.10E), in the assays with the 26bp duplex region RNA substrate. The unwinding reactions were inhibited at higher ATP concentrations for both putative isoforms (3.10, C & E). The decrease in the helicase activity of SCO3550 at elevated ATP concentrations might be due to a reduction in the free Mg^{2+} ions. Mg^{2+} ions are believed to serve as a bridge between the ATP molecules and DExD/H-box proteins (Rocak and Linda 2004). A molar excess of ATP over the Mg^{2+} ions might reduce the amount of the Mg^{2+} ions that interact with the recombinant proteins. The decrease in the helicase activity of SCO3550 at elevated ATP concentrations might also be due to reannealing of the dissociated single-stranded RNAs, however, no experiments were done to verify either of these possibilities. ATP hydrolysis and not just ATP binding was required for the reaction, as the non-hydrolyzable ATP analogue AMP-PNP could not fully substitute for ATP in the reaction (Fig. 3.11, E & G). Basal dissociation of the duplex RNA was observed in the

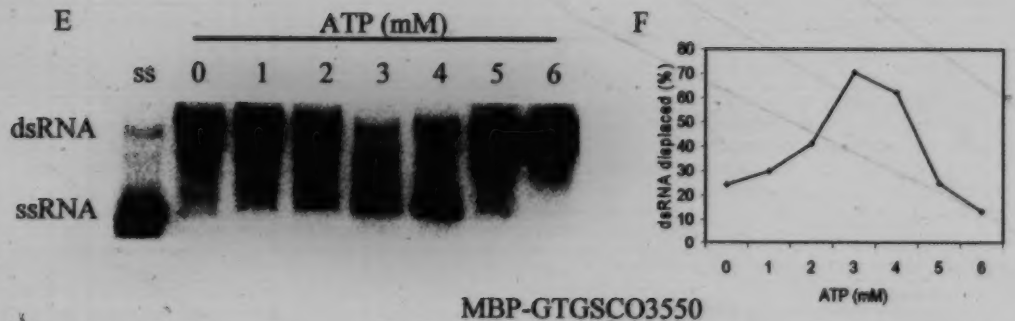
Figure 3.10. Effect of ATP concentration on the 5' to 3' unwinding activity of SCO3550. Influence of the amounts of ATP on 5' to 3' unwinding activities of MBP-ATGSCO3550 (A & C) and MBP-GTGSCO3550 (E). The assays for A were done with 800 ng amount of the MBP-ATGSCO3550 using the substrate shown in Fig. 3.6A. The experiments for A were done twice and a representative result is shown. The assays for C and E were done with 1000 ng of the proteins using the substrate shown in Fig. 3.6B. The experiments for C and E were done three times and representative results are shown. The upper RNA strand of the substrate was radiolabeled in the assays done with MBP-GTGSCO3550, while the lower RNA strand of the substrate was radiolabeled in the assays done with the MBP-ATGSCO3550. The right panels (B, D, and F) are the quantification of the results shown in the left panels. In B and D, the background signal from self-dissociation of the substrate was deducted when percent dsRNA displaced was calculated. The ss in the left panels denotes the boiled substrate control (no protein) and the ds in panels A and C denotes double-stranded RNA substrate incubated in the absence of protein at 37^0C. The unwinding reactions were done as described in section 2.5.2.



MBP-ATGSCO3550

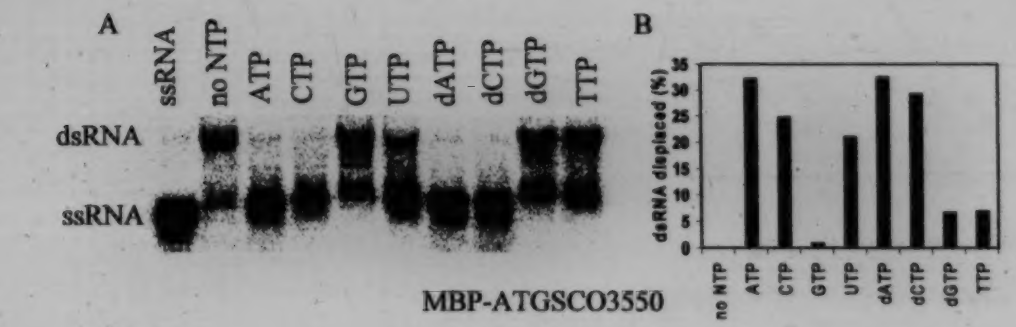


MBP-ATGSCO3550

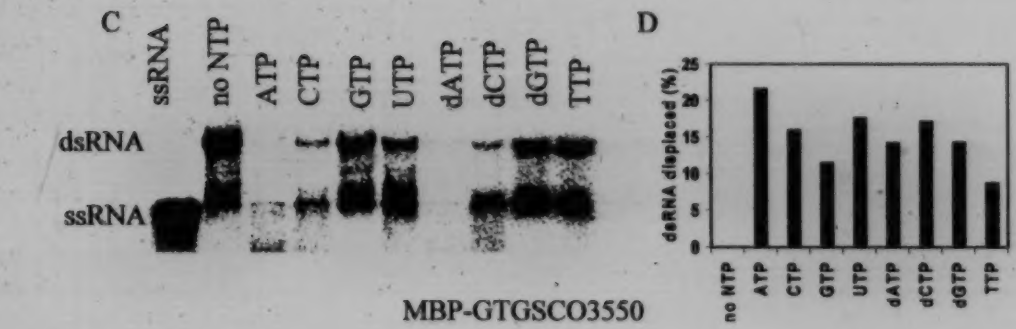


MBP-GTGSCO3550

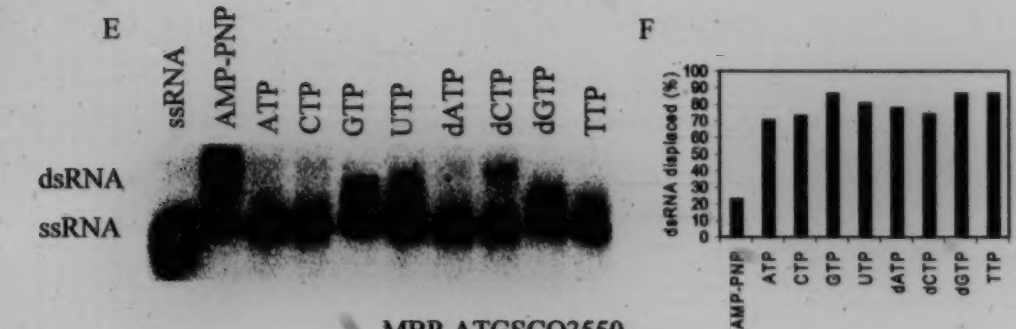
Figure 3.11. NTP requirements for MBP-SCO3550 RNA unwinding activity. Nucleotide dependence of the unwinding reaction for the MBP-ATGSCO3550 (A & E) and MBP-GTGSCO3550 (C & G). The assays for A and B were done with the substrate shown in Fig. 3.6A while those for E and G were done with the substrate shown in Fig. 3.6B. The experiments were done seven times (for A), three times (for C), five times (for E), and two times (for G) and representative results are shown. The right panels, B, D, F, and H, are the quantification results of A, C, E, and G. The background signal corresponding to the no NTP lane was deducted from all values reported in B and D. The assays were performed as described in the Materials and Methods (see section 2.5.2).



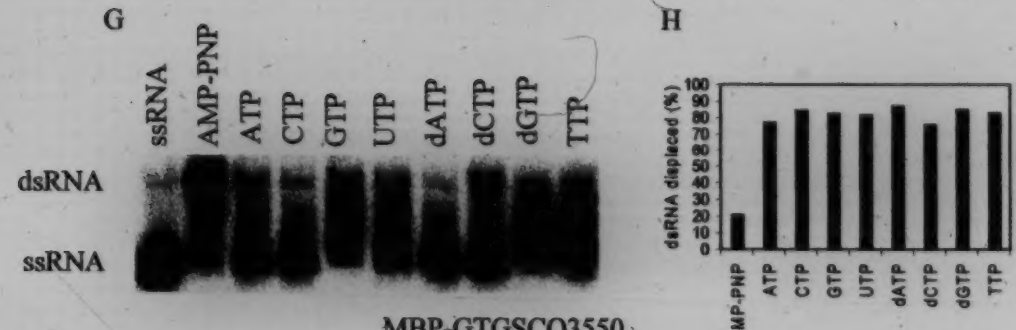
MBP-ATGSCO3550



MBP-GTGSCO3550



MBP-ATGSCO3550

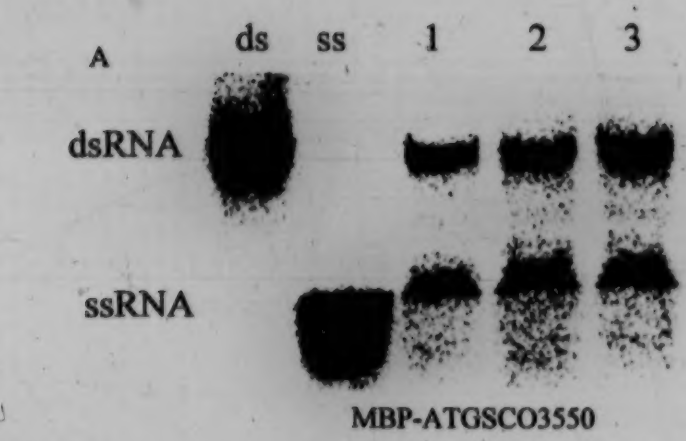


MBP-GTGSCO3550

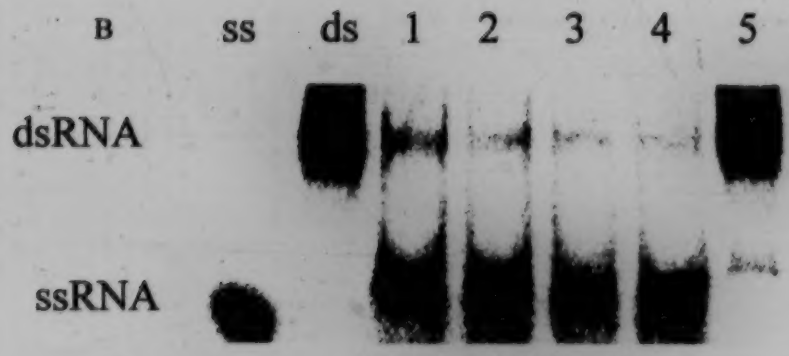
the absence of ATP showing that the artificial ds RNA substrates may be destabilized by thermal fluctuations (Fig. 3.10, A & C, Fig. 3.11, A & C). Alternatively, the low level of observed dissociation in the absence of added ATP could have resulted from cellular ATP being copurified with the proteins. In addition, the unwinding was not catalyzed by nonspecific protein, because GST-BldG (gift from Annie Wong) did not cause unwinding of the artificial dsRNA substrate (Fig. 3.12). Since it is also possible that the observed helicase activity could have resulted from *E. coli* proteins that copurified with either MBP-ATGSCO3550 or MBP-GTGSCO3550, control *E. coli* extracts were run over amylose resin and then tested for unwinding activity. Those extracts did not unwind the duplex RNA indicating that the observed helicase activity could not have come from *E. coli*.

The DExH-box and DEAH-box subfamilies of superfamily II RNA helicases usually have a broad choice for NTPs as the source of energy to unwind double-stranded RNAs (Rocak and Linder 2004). To test this requirement for the recombinant SCO3550 isoforms, the remaining three ribonucleoside triphosphates (CTP, GTP & UTP) and the four deoxyribonucleoside triphosphates (dATP, dCTP, dGTP & TTP) were used for the RNA unwinding assays. The results indicated that all of the common NTPs and dNTPs were utilized for the unwinding activities to an almost equal efficiency by both of the recombinant proteins (Fig.3.11, C, E & G). For an unknown reason, the presence of GTP or dGTP in the unwinding reactions caused smearing of the ssRNA produced (Fig.3.11, E & G). Apart from the nucleotides, more than one divalent cation may support the enzymatic activities of DExD/H-box proteins. The divalent cation requirements for the recombinant SCO3550 proteins were therefore tested. The results in Fig.3.13 indicate that

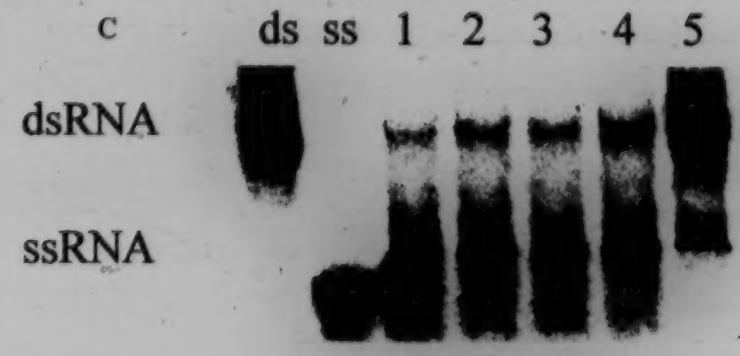
Figure 3.12. GST-BldG does not enhance the unwinding activity of SCO3550. The addition of GST-BldG to the helicase reactions of MBP-ATGSCO3550 (A& B) and MBP-GTGSCO3550 (C). The assays for B and C were done once in the presence of 800 ng of the recombinant SCO3550. Lanes 1 to 4 for panels B and C are 0, 200, 500 and 1000 ng respectively of GST-BldG, in addition to 800 ng of recombinant SCO3550, while lane 5 for panels B and C contained 1000 ng of the recombinant BldG alone. The assay for A was also done once in the presence of 200 ng MBP-ATGSCO3550. Lanes 1 to 3 for panel A are 0, 200 and 500 ng respectively of GST-BldG, in addition to 200 ng MBP-ATGSCO3550. The helicase assays were done as described in the Materials and Methods (see section 2.5.2). The ss in panels A, B, and C denotes single-stranded RNA generated by heating dsRNA substrate at 95^0C for 5 min, while the ds stands for double-stranded RNA.



MBP-ATGSCO3550



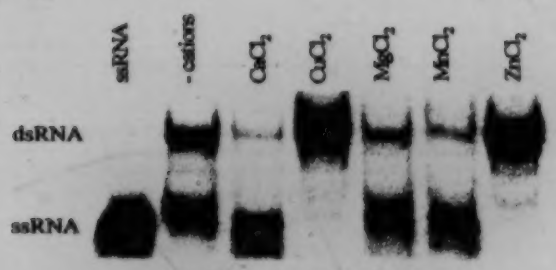
MBP-ATGSCO3550



MBP-GTGSCO3550

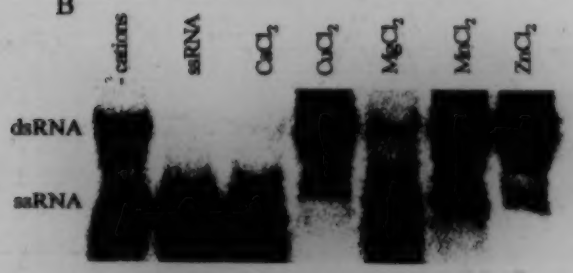
Figure 3.13. Divalent cation requirements for MBP-SCO3550 RNA unwinding activity. Divalent cation dependence of the unwinding reactions for the MBP-ATGSCO3550 (A & C) and MBP-GTGSCO3550 (B & D). The assays were performed as described in the Materials and Methods (see section 2.5.2). The assays for A and B were done with the substrate shown in Fig. 3.6A, while those of C and D were done with the substrate shown in Fig. 3.6B. The experiment was done once with each of the substrates using MBP-GTGSCO3550 and the results are shown. The experiments were done three times with MBP-ATGSCO3550 using each of the substrates and representative results are shown.

A



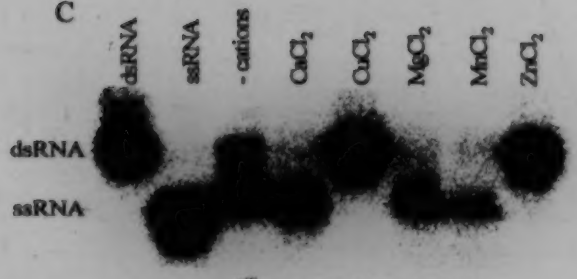
MBP-ATGSCO3550

B



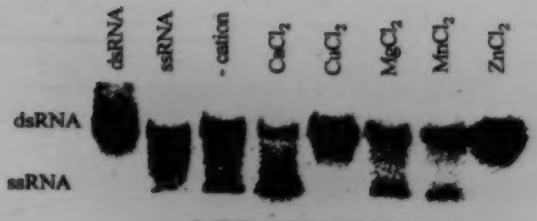
MBP-GTGSCO3550

C



MBP-ATGSCO3550

D

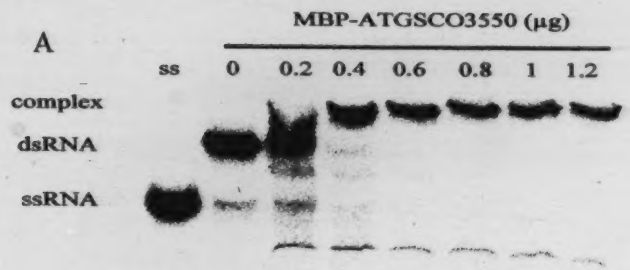


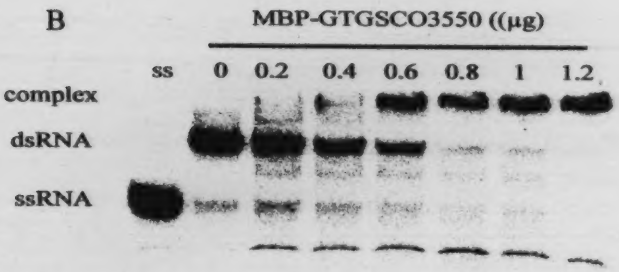
MBP-GTGSCO3550

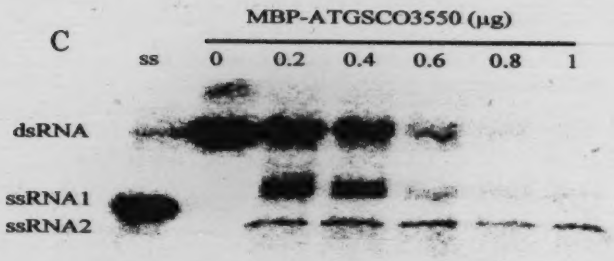
the unwinding activities of the recombinant proteins were supported by $CaCl_2$, $MgCl_2$ and $MnCl_2$ but inhibited by $CuCl_2$ and $ZnCl_2$. The boiled dsRNA control in panel D (labeled ssRNA) was not fully dissociated because of insufficient heating of a pooled 11X reaction.

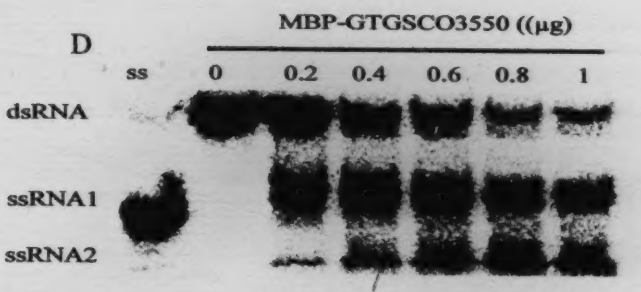
Some DExD/H-box proteins unwind in both 3' to 5' or 5' to 3' directions. These directionalities are determined by whether the helicase binds to a 3' or a 5' tail of the artificial partial dsRNA substrates. From the results described above, it could be concluded that the recombinant SCO3550 unwinds in 5' to 3' direction because the standard dsRNA substrates used for the assays have 5'overhangs. To determine whether SCO3550 also unwinds RNA substrates in the 3' to 5' direction, artificial 3'-tailed dsRNA substrates needed to be constructed (Fig. 3.6, C & D). Several problems were encountered while making the substrate shown in Fig. 3.6C. The substrate was broken down by radiolysis after one week of storage at -20^{0}C. After two attempts, a relatively clean substrate was obtained and used for the assays. Similar reaction conditions described for the 5'-tailed dsRNA substrates were used to test for the 3' to 5' unwinding activity of the protein. As can be seen by the formation of a slower migrating complex, both the MBP-ATGSCO3550 and MBP-GTGSCO3550 bound to the substrate after incubation at 37^{0}C for 30 min (Fig. 3.14, A & B). Even though this result was not expected because the reaction conditions used are for unwinding rather than RNA binding, it demonstrated that SCO3550 binds to RNA in the presence of ATP. It was shown in the previous study that the recombinant ATGSCO3550 destabilized dsRNA to some extent in a 3' to 5' direction (Gislason 2005). Therefore, failure of the recombinant protein to unwind the 3'-tailed substrate in this study might be due to inherent resistance

Figure 3.14. Influence of enzyme concentration on 3' to 5' unwinding activity of SCO3550. The effect of increasing amounts of MBP-ATGSCO3550 (A & C) and MBP-GTGSCO3550 (B & D) on 3' to 5' unwinding activity. The assays for A and B were done with the substrate shown in Fig. 3.6C while the assays for C and D were done with the substrate shown in Fig. 3.6D. Samples for A and B were run on 10% native polyacrylamide gels, while those for C and D were run on 10% denaturing polyacrylamide gels. The lane ss in all panels represents boiled substrate control (no protein added). The reaction conditions were as described in the Materials and Methods (see section 2.5.2).


A
MBP-ATGSCO3550 (µg)
ss 0 0.2 0.4 0.6 0.8 1 1.2
complex
dsRNA
ssRNA


B
MBP-GTGSCO3550 ((µg)
ss 0 0.2 0.4 0.6 0.8 1 1.2
complex
dsRNA
ssRNA


C
MBP-ATGSCO3550 (µg)
ss 0 0.2 0.4 0.6 0.8 1
dsRNA
ssRNA1
ssRNA2


D
MBP-GTGSCO3550 ((µg)
ss 0 0.2 0.4 0.6 0.8 1
dsRNA
ssRNA1
ssRNA2

from the substrate. Even though the substrate appeared to be of high quality after synthesis, several unwanted bands appeared after addition of the test protein (Fig. 3.14, A & B). It has been reported that use of DNA templates with 3' overhangs to synthesize artificial RNA substrates sometimes produces extraneous transcripts in addition to those expected (Schenborn and Mierendorf 1985). These extraneous transcripts may contain sequences complementary to the expected transcripts required for the preparation of the artificial dsRNA substrate. Although the *Hae* II-restricted plasmid used to produce the radiolabeled transcript was treated with T4 DNA polymerase (Fermentas) to generate a blunt end, the reaction might not have been 100% efficient and this might have contributed to the extra bands observed after the helicase reactions. While the appearance of these bands suggested a very low level of RNA destabilizing activity, it made interpretation of the result difficult. Therefore to avoid this, *Pvu* II was used in place of *Hae* II to generate a blunt end DNA template for the synthesis of the 3'-tailed substrate shown in Fig.3.6D. The treatment of this substrate with different amounts of MBP-GTGSCO3550 resulted in a gradual decrease in the dsRNA substrate and accumulation of two RNA species (Fig. 3.14D, labeled ssRNA 1&2). The faster migrating RNA species (ssRNA2) may represent an alternate single-stranded conformer. Alternatively, the RNA species may be a stable product obtained as a result of cleavage by RNase copurifying from *E. coli*. A similar trend was observed for the MBP-ATGSCO3550 (Fig. 3.14C), indicating that both forms of SCO3550 unwind duplex RNA in the 3' to 5' direction.

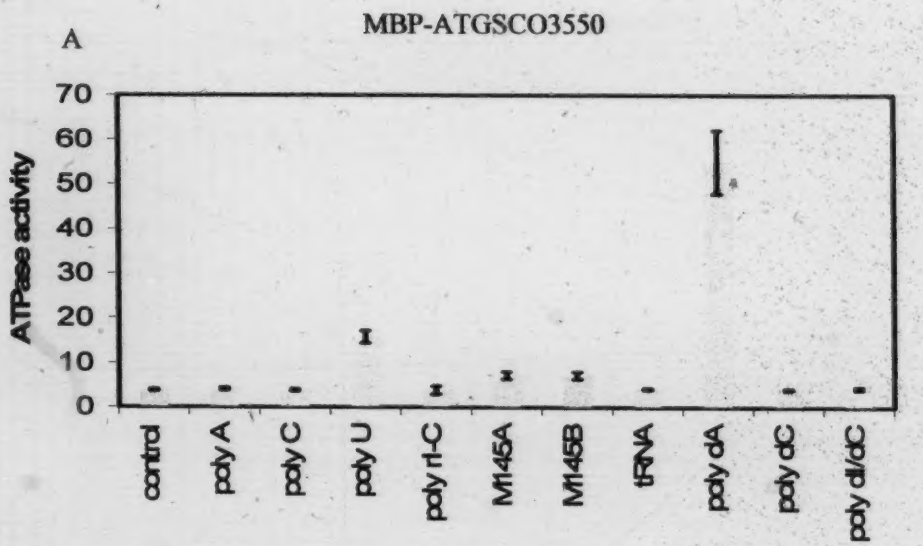
3.3. Hydrolysis of NTPs by SCO3550

RNA helicases possess nucleic acid-stimulated NTPase activities. To test this ability for SCO3550, the coupled spectrophotometric method (Iost et al. 1999) was used. In this experiment, the hydrolysis of NTP leads to the production of NDP. In the presence of NDP, pyruvate kinase converts phosphoenol pyruvate to pyruvate. Lactate dehydrogenase then catalyzes the synthesis of lactate from pyruvate using NADH as a coenzyme. Thus, the method links NTP hydrolysis to the oxidation of NADH which can be monitored by measurement of its absorbance at 338 nm.

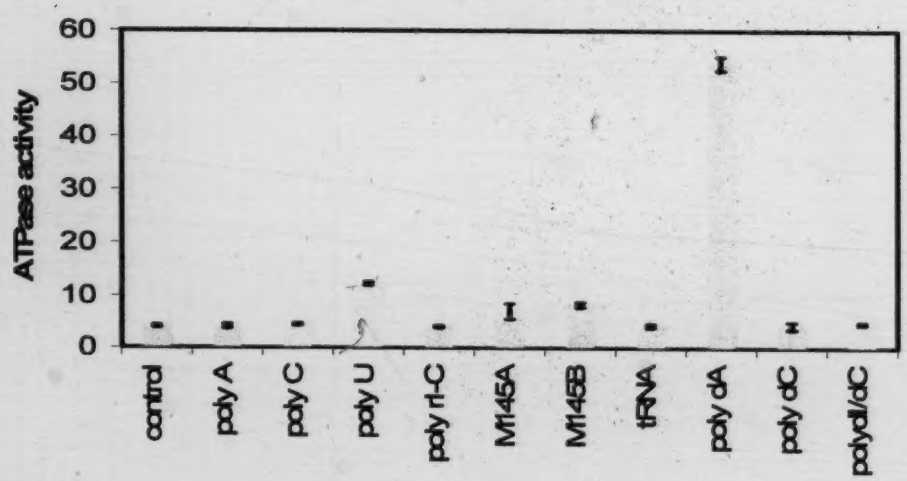
The enhancement of ATPase activity of the ATG and GTG-versions of SCO3550 by various nucleic acids was tested. The different kinds of nucleic acids commonly used to test for the nucleic acid-stimulated NTPase activities of DExD/H-box proteins were used and include: poly A, poly C and poly U (represent ssRNA); poly rI-rC (represents dsRNA); poly dA and poly dC (represent ssDNA); poly dI/dC (represents dsDNA); total RNAs from *Streptomyces coelicolor* M145 strain (Gift from Kim Colvin); and tRNA from *E. coli*. The experiments were done as described in the Materials and Methods (see section 2.5.1). The results indicate that the recombinant MBP-tagged SCO3550 isoforms hydrolyzed ATP at low level in the absence of nucleic acids. However, in the presence of total RNAs from *Streptomyces coelicolor* M145 strain, poly U and poly dA, the ATPase activity was enhanced by at least 1.7 fold (Fig. 3.15). A comparison of Figs. 3.15A and B shows that the nucleic acids stimulated the ATPase activities of both MBP-ATGSCO3550 and MBP-GTGSCO3550 to similar degrees. For instance, the measured values for the kcat of ATP hydrolysis by the recombinant ATG-SCO33550 and GTG-SCO3550 were 55.0/min and 53.8/min, respectively (Fig. 3.15, see Appendix).

Figure 3.15. Influence of nucleic acids on the ATPase activities of MBP-SCO3550. (A) The effect of nucleic acids on the ATPase activity of recombinant ATG-SCO3550. (B) The effect of nucleic acids on the ATPase activity of recombinant GTG-SCO3550. The control in both cases had no nucleic acid. M145A and M145B are the total RNAs harvested from the M145 strain of *S. coelicolor* grown on a solid medium for 18 and 24 hours, respectively. The reactions were set up in triplicate as described in the Materials and Methods (see section 2.5.1). The experiments were done once using each of the recombinant proteins. The total RNA was used at a concentration of 300µg/mL while the rest of the nucleic acids were used at a concentration of 100µg/mL. The ATPase activities were measured in µmol/min/µmol enzyme.

A


MBP-ATGSCO3550
ATPase activity
70
60
50
40
30
20
10
0
control
poly A
poly C
poly U
poly rI-C
M145A
M145B
tRNA
poly dA
poly dC
poly dI/dC

B

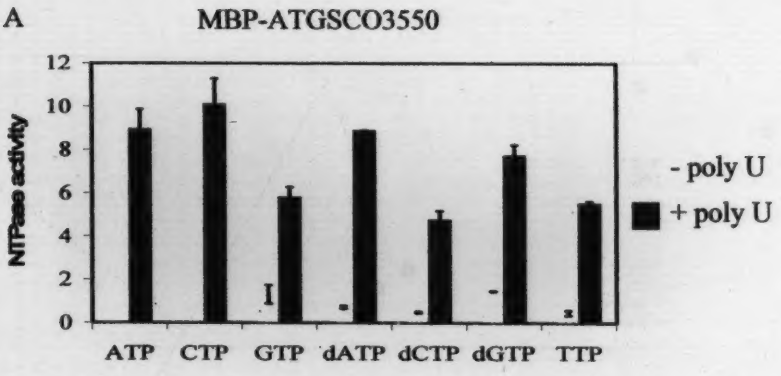

MBP-GTGSCO3550
ATPase activity
60
50
40
30
20
10
0
control
poly A
poly C
poly U
poly rI-C
M145A
M145B
tRNA
poly dA
poly dC
polydI/dC

Interestingly, total RNAs from *S. coelicolor* stimulated the ATPase activities of both proteins less than poly U and poly dA (Fig. 3.15). The rest of the nucleic acids did not stimulate the ATPase activities of the proteins since the kcat values (approximately 4.0/min) of ATP hydrolysis were similar to the control with no RNA (Fig. 3.15, see Appendix).

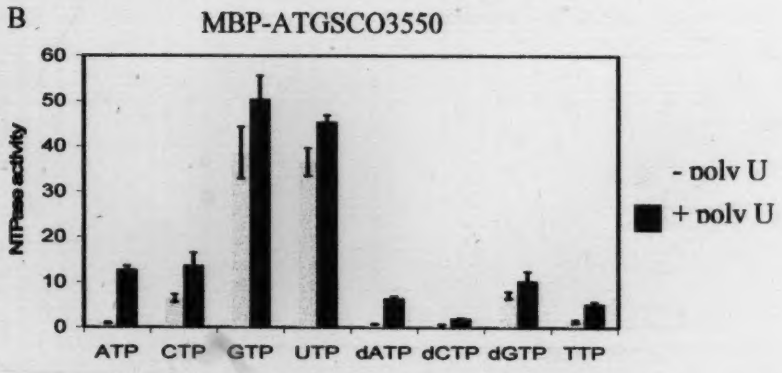
As shown in section 3.2 all of the common NTPs and dNTPs supported the duplex RNA unwinding activity of SCO3550. It was therefore of interest to see if the hydrolysis of those same NTPs and dNTPs by SCO3550 would be enhanced by the addition of nucleic acid. Additionally, many other DExH/DEAH-box RNA helicases have been shown to demonstrate this ability (Rocak and Linder, 2004). To determine if SCO3550 follows the same trend, the eight common NTPs and dNTPs were used for NTPase hydrolysis assays. The experiments were done with poly U even though poly dA gave the maximum stimulation of the ATPase activity because it is less expensive. The results showed that poly U enhances hydrolysis of all of the NTPs and dNTPs by SCO3550 (Fig. 3.12, A, B & C). The recombinant MBP-ATGSCO3550 hydrolyzed most of the nucleotides significantly in the absence of poly U in the first experimental trial (Fig. 3.16B). In the second experiment however, the NTP hydrolysis by the recombinant MBP-ATGSCO3550 in the presence or absence of poly U was more distinct (Fig. 3.16A). Additionally, for an unknown reason, the recombinant protein failed to hydrolyze UTP in the presence or absence of RNA in the second experiment. Nonetheless, it could be said that the recombinant ATG-SCO3550 hydrolyzes UTP since that was observed in the first experiment and it is capable of hydrolyzing the deoxyribonucleoside counterpart (TTP) in the presence of poly U (Fig. 3.12, A&B). With the exception of reactions

Figure 3.16. NTP hydrolysis by MBP-SCO3550. RNA-dependent NTPase activities of the MBP-ATGSCO3550 (A&B) and MBP-GTGSCO3550 (C). Poly U (100μg/mL) was used as the RNA substrate for these experiments in the presence of 1000 ng of the recombinant proteins. The reactions were set up in triplicate as described in the Materials and Methods (see section 2.5.1) and the assays performed once. The NTPase activities were measured in μmol/min/μmol enzyme.

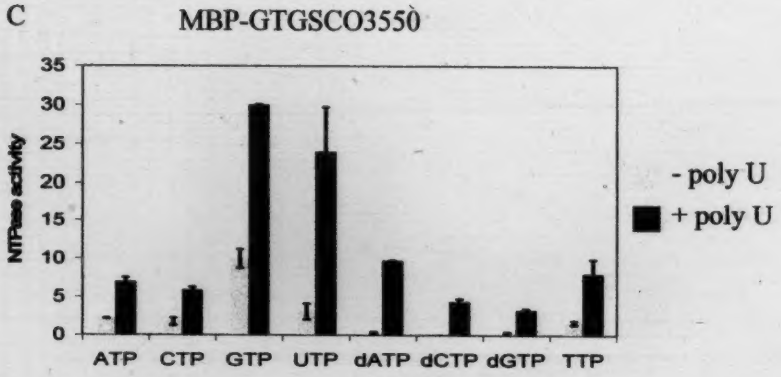
A


MBP-ATGSCO3550
NTPase activity
12
10
8
6
4
2
0
ATP
CTP
GTP
dATP
dCTP
dGTP
TTP
- poly U
+ poly U

B


MBP-ATGSCO3550
NTPase activity
60
50
40
30
20
10
0
ATP
CTP
GTP
UTP
dATP
dCTP
dGTP
TTP
- polv U
+ polv U

C


MBP-GTGSCO3550
NTPase activity
35
30
25
20
15
10
5
0
ATP
CTP
GTP
UTP
dATP
dCTP
dGTP
TTP
- poly U
+ poly U

containing GTP and UTP, comparable kcat values for poly U-stimulated NTP hydrolysis were obtained for each nucleotide in the first and second experiments: ATP (12.8/min, 9.0/min), CTP (13.7/min, 10.1/min), dATP (6.4/min, 8.9/min), dCTP (2.0/min, 4.8/min), dGTP (10.3/min, 7.7/min), and TTP (5.3/min, 5.5/min) (Fig. 3.16, A&B, see Appendix). The slight difference in the enzymatic activities of the recombinant MBP-ATGSCO3550 in the first and the second experiments might be due to the fact that the protein was purified from different cultures of the expression host or handled differently during or after the purification. It was no surprise that the NTPase hydrolysis by the recombinant MBP-GTGSCO3550 is similar to the ATG-version (Fig. 3.16) since they share several similar biochemical activities described previously. To conclude, the overall results suggest that the recombinant SCO3550 isoforms possess nucleic acid-dependent NTPase activity.

CHAPTER 4:
DISCUSSION

4.0. DISCUSSION

SCO3550 has been described as a putative RNA helicase based on the presence of seven conserved motifs similar to those found in the members of superfamily II of RNA helicases (Stoehr 2001). In previous studies, SCO3550 has been shown to be expressed as two putative isoforms. These isoforms have been speculated to result from two predicted in-frame translational start codons (Gislason 2005). In this work, it was shown that the maltose binding protein-tagged SCO3550 isoforms possess NTP-dependent RNA unwinding activities and nucleic acid-dependent NTPase activities. Additionally, the enzymatic activities of the recombinant SCO3550 isoforms were similar.

SCO3550 utilizes any of the eight common NTPs (ATP, CTP, GTP, UTP, dATP, dCTP, dGTP, and TTP) with nearly equal efficiency. This broad choice for nucleoside triphosphates is also shared by several studied DExH-box and DEAH-box RNA helicases, which are known to be promiscuous in their choice for NTPs (Rocak and Linder 2004). Conversely, the DEAD-box RNA helicases use mainly ATP/dATP (Cordin et al. 2006). Like SCO3550, *Drosophila* Maleless protein (MLE) required for X chromosome dosage compensation, utilizes each of the eight common NTPs in a helicase activity with no selectivity (Lee et al. 1997). MLE is a homologue of the human RNA helicase A (RHA) and shares almost identical biochemical activities with it (Lee et al. 1997). RHA also unwinds dsRNA substrates using the energy derived from any of the NTPs with no preference (Lee and Hurwitz 1992). A homologue of RHA and MLE, DNA helicase II from calf thymus nuclei, has also been described to use all the four NTPs and dNTPs although ATP and dATP were preferred to other nucleotides (Zhang and Grosse 1991). This characteristic of broad choice for the nucleoside triphosphates by

SCO3550 is also exhibited by some DExH-box proteins from viruses. The prototypical viral DExH-box helicase NPH-II from vaccinia virus (Shuman 1992), NS3 from hepatitis C (Wardell et al. 1999) and the cylindrical inclusion protein from plum pox virus (Lain et al. 1990) have been described to utilize all four NTPs and dNTPs. Additionally, three characterized DEAH-box splicing factors from yeast (Prp2, Prp16 and Prp22) hydrolyze all the eight common nucleoside triphosphates (Kim et al. 1992; Wang et al. 1998; Tanaka and Schwer 2005). In contrast to most characterized DExH/DEAH-box proteins, the human splicing factor U5-200kD hydrolyzes only ATP, CTP and their deoxyribonucleoside counterparts, while a DExH-box DNA helicase from *Saccharomyces cerevisiae*, Rad3, hydrolyzes only ATP and dATP (Laggerbauer et al. 1998; Naegeli et al. 1992).

The kcat values for ATP hydrolysis in the presence of poly dA for both isoforms of SCO3550 is approximately 54/min. This value is the same as that reported for RNA helicase A from HeLa cells (54/min) (Lee and Hurwitz 1992) and also quite close to that of a human nuclear RNA helicase, p68 (45/min) (Hirling et al. 1989). The ATPase activity of SCO3550 may, however, be considered as relatively low when comparing the obtained kcat value of 54/min to the reported kcat values of several other DExD/H-box proteins. For example, the SCO3550 kcat value is at least five times smaller than that of Prp22p (400/min) and Ded1p (340-680/min) from yeast (Wagner et al 1998; Iost et. al 1999), DbpA from *E. coli* (600/min) (Tsu et al. 2001) and CrhR from cyanobacteria (500/min) (Chamot et al. 2005). On the other hand, the obtained kcat value for SCO3550 is at least seven times larger than that of the elF4A (3.0 /min) and Has1p from yeast (5.5/min) (Lorsch and Herschlag 1998; Rocak et al. 2005), An3 of Xenopus (6/min)

(Gururajan and weeks 1997), human DEAD-box protein Ddx42p (2/min) (Uhlmann-schiffler et al. 2006), RNA helicase II (1.9/min) (Flores-rozas and Hurwitz 1993) and SrmB of *E coli* (1.2/min) (Nishi et al. 1988).

Unlike DbpA, Ded1, Has1 and p68 that hydrolyze NTPs strictly under the influence of RNA (Tsu et al. 2001; Iost et al. 1999; Rocak et al. 2005; Hirling et al. 1989), SCO3550 demonstrates basal hydrolysis of most of the NTPs in the absence of RNA. The ATPase activity of SCO3550 is stimulated by both RNA and DNA. The stimulation of ATPase activities with both RNA and DNA is demonstrated for several DExH/DEAH-box proteins including vaccinia virus NPH-II protein (Shuman 1993), hepatitis C virus NS3 protein (Preugschat et al. 1996), RNA helicase A from HeLa cells (Lee and Hurwitz 1992), and DNA helicase II from calf thymus tissue (Zhang and Grosse 1991). Interestingly, the ATPase activities for both SCO3550 and RNA helicase A are stimulated better by single-stranded DNA homopolymers than by RNAs. However, whereas poly dA stimulates ATPase activity of SCO3550, it does not support the ATPase activity of RNA helicase A. The ATPase activity of RNA helicase A was enhanced maximally by poly dT and poly dI (Lee and Hurwitz 1992), which were not tested for SCO3550. For several characterized RNA helicases, the stimulation of their ATPase activities by RNA or DNA suggests whether they can bind to RNA or DNA. This relationship between RNA/DNA stimulation of ATPase activities and RNA/DNA binding of RNA helicases is discussed further below.

SCO3550 unwinds dsRNA duplexes in an NTP-dependent manner. This result contrasts the ATP-independence previously observed with MBP-ATGSCO3550 (Gislason 2005), however, NTP-dependence was reproducibly observed in this study

using both the ATG and GTG-versions of the protein. In all experiments performed in this study, SCO3550 was required in a large amount to unwind the dsRNA substrates (see section 6.3). This raises the possibility that the unwinding may not be a true catalytic activity. Under such conditions, SCO3550 may just occupy ssRNA generated from thermal fluctuations. However, unlike the DEAD-box proteins Dbp9p of yeast, DbpA and CsdA of *E. coli* that stoichiometrically destabilize dsRNA without the use of ATP (Kikuma et al. 2004; Boddeker et al. 1997; Jones et al. 1996), SCO3550 unwinding activity is energy-dependent because the non-hydrolyzable ATP cannot fully support the unwinding activity. SCO3550 dsRNA unwinding activity is therefore likely to be enzymatic rather than stoichoimetric. The use of an excess molar amount of protein over substrate to unwind RNA duplex is not peculiar only to SCO3550. The Ded1p and Prp16p from yeast, (Iost et al. 1999; Wang et al. 1998), RNA helicase II (Flores-Razas and Hurtwitz 1993), p68 (Hirling et al. 1989), Upf1p (Czaplinski et al. 1995) and elF4A (Rozen et al. 1990; Pause and Sonenberg 1992) are all required in a large amount to achieve unwinding activities. Interestingly, only a few RNA helicases including vaccinia virus NPH-II, RNA helicase A from HeLa cells and the splicing factor Prp22, have been described to act with the catalytic amount of the protein over the substrate (Shuman 1992; Lee and Hurwitz 1992; Tanaka and Schwer 2005). These helicases may have high turnover numbers for the unwinding reactions. Alternatively, with regards to a particular substrate used, the dissociated strands might fold intramolecularly, preventing their reannealing (Iost et al. 1999).

Clearly, the unwinding activities described for SCO3550 in this work are energy dependent. As stated above, Gislason (2005) previously showed that SCO3550

destabilized duplex RNA in the presence or absence of ATP. The difference in the unwinding activities of the current and previous study might be due to the use of a relatively old artificial dsRNA substrate in the previous study. Temperature fluctuations contribute to the dissociation of a substrate; hence a prolonged storage may affect the integrity of the substrate, especially substrate with only 14 bp RNA duplex region. Additionally, based on the fact that SCO3550 has a significant basal unwinding activity in the absence of ATP, substrates with low quality could be fully dissociated even in the absence of an energy source.

Mg^{2+} facilitates binding of ATP to DExD/H-box proteins. This role of the Mg^{2+} can be substituted for by other divalent cations depending on the particular helicase protein. In this study, the 5' to 3' unwinding activity of SCO3550 was supported by $CaCl_2$, $MgCl_2$ and $MnCl_2$ but inhibited by $CuCl_2$ and $ZnCl_2$. In the absence of divalent cations, SCO3550 also displayed significant RNA unwinding activity. This characteristic is similar to that of DNA helicase II, whose unwinding activity is supported by $MgCl_2$ and $MnCl_2$, but exhibited significant unwinding activity even in the absence of these divalent cations (Zhang and Grosse 1991). In contrast to the divalent cation requirements of SCO3550, $CaCl_2$ and $MnCl_2$ cannot substitute for $MgCl_2$ in the unwinding reactions of RNA helicase A (Lee and Hurwitz 1992).

The DExD/H-box proteins are described to have 5' to 3' unidirectionality, 3' to 5' unidirectionality, and bidirectionality, depending on the requirement for the substrates with a 5'-tail, a 3'-tail, or both. SCO3550 unwinds duplexes with 5' or 3' overhangs. The prototypical RNA helicase elF4-4A (Rozen et al. 1990), nuclear RNA helicase, p68, (Huang and Liu 2002), yeast Has1p RNA helicase (Rocak et al. 2005), and

cyanobacterial CrhR RNA helicase (Chamot et al. 2005) are among the few DEAD-box proteins that unwind artificial dsRNA substrates in both directions. Most previously studied DEAH/DExH-box RNA helicases unwind in a 3' to 5' direction (Tanaka and Schwer 2005; Tai et al. 1996; Shuman 1993; Lee and Hurwitz 1992; Lee et al. 1997; Zhang and Grosse 1994). The only DExH-box protein that does not unwind in the 3' to 5' direction is the DNA helicase Rad3 whose polarity is strictly 5' to 3' (Naegeli et al. 1992). Like SCO3550, the splicing factor Prp16, a DEAH-box RNA helicase, is bidirectional but it unwinds with less efficiency in the 5' to 3' direction (Wang et al. 1998).

The in vitro enzymatic activities of SCO3550 are relatively low. This may be due to a low intrinsic catalytic capability of the protein. Additionally, it may be due to the absence of a cofactor protein. The viral DExH-box protein NS3 displays poor in vitro RNA unwinding activity, however its activity is enhanced when it is in a complex with NS4A (Pang et al. 2002). The eukaryotic initiation factor, elF4A, is a non-processive RNA helicase. In the presence of elF4B or elF4H, elF4A unwinds longer, more stable duplexes with both an increase in initial rate and maximum amount of the duplex unwound (Rogers et al. 1999). The requirement for a cofactor to enhance helicase activity has also been shown for a human DEAD-box protein, Dddx42p (Uhlmann-Schiffler et al 2006). Due to the numerous predicted links between SCO3550 and BldG, it was speculated that BldG could be a protein cofactor of SCO3550. However, the addition of a recombinant BldG to the unwinding reactions of SCO3550 failed to promote the helicase activity of SCO3550. It is worth mentioning that freezing of recombinant SCO3550 affects its enzymatic activities. In this context it is also possible that BldG may be inactivated after freezing, hence its inability to stimulate SCO3550 unwinding activity.

However, it is also just as likely that SCO3550 may require a protein other than BldG as a cofactor. The low enzymatic activities of the recombinant SCO3550 isolated from *E. coli* may also be due to lack of post-translational modifications such as phosphorylation and methylation. There is also the possibility that the protein could be partially misfolded because it was purified as a recombinant protein (Cordin et al. 2006).

Several possible roles of SCO3550 in *Streptomyces coelicolor* have been discussed previously (Stoehr 2001). It has been suggested that SCO3550 may promote the degradation of the *bldG* monocistronic transcript by RNase through destabilizing the secondary structures at the 3' terminus of the transcript. It has also been proposed that SCO3550 may unwind the secondary structures between *bldG* and *orf3* on the polycistronic transcript and permit the translation of the latter. In agreement with these hypotheses, SCO3550 was shown in the previous (Gislason 2005) and current studies to possess bidirectional RNA unwinding activity. It is possible that SCO3550 could use its 3' to 5' unwinding activity to promote the degradation of the *bldG* monocistronic transcript and its 5' to 3' unwinding activity in the translation of *orf3*. These predicted roles of SCO3550 in mRNA degradation and translation would not be unusual because they have been described for an RNA helicase, Upf1, from *Saccharomyces cerevisiae* even though its function in mRNA decay is not fully understood (Leeds et al. 1992; Cui et al. 1995; Czaplinski et al. 1995). As well, some RNA helicases have been shown to play multiple roles in the cell. For example, the prototypical DExH-box protein, NPH-II, has been implicated in mRNA transport, transcription termination and remodeling of ribonucleoproteins (Gross and Shuman 1996; Fairman et al. 2004). The DEAH-box RNA

helicase A is involved in translation and transcription (Nakajima et al. 1997; Tetsuka et al. 2004; Hartman et al. 2006).

Another predicted role of SCO3550 is in the regulation of the transcription of *orf3* using its unwinding activities. It has been suggested previously that the stem-loops in the *bldG-orf3* transcript could be serving as a rho-independent termination signal that might prevent the transcription of *orf3*. SCO3550 has been speculated to destabilize the stem-loops to allow the transcription of *orf3* (Stoehr 2001). Support for this idea comes from the finding that the ATPase activity of SCO3550 is stimulated by a DNA homopolymer. Interestingly, the stimulation of ATPase activities of DExD/H-box proteins by DNA/RNA indicates their ability to bind to DNA/RNA and in several instances their ability to destabilize duplex DNA/RNA. The DECH-box helicase, NS3 from hepatitis C virus, binds to both RNA and DNA and has an ATPase activity that is stimulated by both RNA and DNA homopolymers (Gwack et al. 1996; Preugschat et al. 1996). Consequently, NS3 destabilizes both RNA and DNA duplexes in an energy-dependent manner (Tai et al. 1996; Pang et al. 2002). Additionally, the DExH-box protein from vaccinia virus has an ATPase activity that is stimulated by nucleic acids (Shuman 1993). In accordance, it binds to DNA and RNA and unwinds duplex DNA and RNA in the presence of NTPs (Shuman 1993; Bayliss and Smith 1996). The *Drosophila* DExH-box protein, maleless (MLE), and its mammalian homologues RNA helicase A and nuclear DNA helicase II, unwind duplex DNA/RNA substrates (Lee et al. 1997; Zhang and Grosse 1994). The nucleic acid-stimulated ATPase activities and nucleic acid binding characteristics have been described for RNA helicase A and nuclear DNA helicase II (Lee and Hurwitz 1992; Zhang and Grosse 1994). The large T antigen, a regulatory

protein from simian virus 40, displays DNA helicase activity at the expense of ATP/dATP and RNA helicase activity at the expense of CTP/dCTP, GTP/dGTP and UTP/TTP (Scheffner et al. 1989). Accordingly, the large T antigen binds to DNA and possesses DNA-stimulated ATPase and RNA-stimulated UTPase activities (Spillman et al. 1979; Giacherio and Hager 1979; Scheffner et al. 1989). The Upf1 protein of *Saccharomyces cerevisiae* demonstrates similar characteristics including the unwinding of DNA and RNA duplexes using the eight common NTPs, albeit with unequal efficiency (Czaplinski et al. 1995). Thus, for many characterized DExD/H-box proteins, DNA-stimulated ATPase activities indicate duplex DNA unwinding.

Interestingly, apart from Upf1 and the large T antigen, the other proteins mentioned above use all of the four NTPs and dNTPs with almost an equal efficiency in the RNA/DNA helicase activities. The nucleotide requirements of these proteins are therefore similar to that of SCO3550. Moreover, with the exception of Upf1 and the large T antigen, the rest of the proteins are DEAH/DExH-box proteins. To the best of my knowledge, all characterized DEAH/DExH-box proteins that have DNA-stimulated ATPase activities and low selectivity for nucleotides, destabilize DNA duplexes when tested. It can therefore be said with a high degree of confidence that SCO3550 unwinds duplex DNA substrates. If this is true, SCO3550 may be directly involved in remodeling of *S. coelicolor* chromosome thereby permitting the transcription of *bldG, orf3,* or a different gene(s). It is also possible that SCO3550 may contribute indirectly to gene transcription by acting as a bridge between transcriptional factors as observed for RNA helicase A (Nakajima et al. 1997). Obviously, experimental proof will be required to back up these statements.

If the similarities and differences in the biochemical activities of DExD/H-box proteins can give clues regarding their cellular functions, then SCO3550 may also be involved in remodeling of ribonucleoproteins as described for the vaccinia virus NPH-II (Fairman et al. 2004). However, because it displays energy-dependent unwinding activities, SCO3550 is unlikely to participate in ribosome biogenesis as described for the *E. coli* DEAD-box protein, CsdA, whose unwinding activities are energy-independent (Toone et al. 1991; Charollais et al. 2004).

The assignment of numerous roles to SCO3550 in the development of *S. coelicolor* may be more convincing if it is truly expressed as isoforms. Although two bands were repeatedly observed by western analysis (Gislason 2005), it is not yet clear that the two bands result from translation from the two possible translation start codons. Translation from a single start codon and processing at the 3' end would also generate two protein bands on the SDS-PAGE gels. In this study, the two putative isoforms of SCO3550, which reflected translation initiation at the GTG or the ATG start codons, exhibited similar biochemical activities. This, together with the fact that the expression pattern in *S. coelicolor* showed that the larger isoform was expressed during early vegetative growth to the time of aerial hyphal formation, and the smaller isoform was expressed during early vegetative growth to the time of sporulation, it is possible that the two isoforms could play similar roles at different developmental stages of the bacterium. However, it is also possible that the putative isoforms may play different roles during the period that they are expressed simultaneously during the life cycle of *S. coelicolor*.

The exact cellular roles of SCO3550 can only be deduced if its cellular protein binding partners and RNA targets are identified. So far immunoprecipitation assays to

pull down the protein binding partners for mass spectrometric analysis have been done with no success. As an alternative strategy, a MBP-pull down experiment could be done and coupled with mass spectrometric analysis to identify any putative binding partners. It would also be interesting to see if SCO3550 interacts with *S. coelicolor* chromosomal DNA. This would provide some evidence in support of a role in the remodeling of the chromosome, and could be accomplished by performing chemical crosslinking experiments. A suggestive role of SCO3550 in transcription could be determined by testing its ability to unwind duplex DNA.

CHAPTER 5:
REFERENCES

5.0. REFERENCES

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CHAPTER 6:
APPENDIX

6.0. APPENDIX

Table 6.1. RNA-dependent NTPase activities of recombinant ATG-SCO3550 (Experiment 1)

	Samples without poly U			Samples without poly U		
NTP	A	T.O./min	Aveg. T.O./min	A	T.O./min	Aveg. T.O./min
	0.189	0.8		3.008	12.4	
	0.229	0.9		3.333	13.8	
ATP	0.265	1.1	0.9	2.924	12.1	12.8
	1.811	7.5		3.11	12.9	
	1.409	5.8		4.11	17.0	
CTP	1.526	6.3	6.6	2.727	11.3	13.7
	10.428	43.1		10.933	45.2	
	7.67	31.7		13.455	55.6	
GTP	9.93	41.0	38.6			50.4
	8.56	35.4		10.545	43.6	
	8.248	34.1		11.276	46.6	
UTP	9.741	40.3	36.6	11.13	46.0	45.4
	0.205	0.8		1.477	6.1	
	0.172	0.7		1.7	7.0	
dATP			0.8	1.5	6.2	6.4
	0.131	0.5		0.507	2.1	
	0.182	0.8		0.48	2.0	
dCTP			0.6	0.475	2.0	2.0
	1.939	8.0		3.071	12.7	
	1.751	7.2		2.058	8.5	
dGTP	1.588	6.6	7.3	2.368	9.8	10.3
	0.429	1.8		1.233	5.1	
	0.34	1.4		1.423	5.9	
TTP	0.282	1.2	1.4	1.171	4.8	5.3

dA/dt is the change of absorbance per unit time. It is measured as milliabsorbance/min.

T.O. is the turnover number of the enzyme.

Sample calculation of the turnover number

From Beer-Lamberts's law,

Absorbance $A = \varepsilon * c * l$ (1)

ε is the extinction coefficient of the absorbing substance, for NADH used in this experiment it is approximately 6300 L/mol/cm at a wavelength of 338 nm.

c is the concentration of the substance expressed in mol/L

l is the path length of the sample. This path length is automatically calculated as 1 cm for the plate reader used to measure the absorbance in this experiment.

From equation (1)

$dA/dt = dc/dt * \varepsilon$, since l is 1 cm

This implies, $dc/dt = dA/dt/\varepsilon$ (2)

Where dc/dt is the rate of reaction

But enzyme activity = moles converted per unit time = rate* reaction volume (V) (3)

Substituting equation (2) into (3),

Enzyme activity = $dA/dt/\varepsilon * V$ (4) V is measured in L

Specific activity of the enzyme = moles converted per unit time per unit mass of the enzyme = enzyme activity / the actual mass (m) of the enzyme present (5)

Substituting equation (4) into (5)

Specific activity = $dA/dt/\varepsilon * V/m$ (6)

Turnover number = moles of substrate converted per unit time / moles of enzyme

= specific activity * molar mass (M) of enzyme (7)

Substituting equation (6) into (7)

Turnover number = $dA/dt/\varepsilon * V/m * M$ (8)

The unit of the turnover number (kcat) is μmol/min/μmol enzyme or usually per min

In the above experiment, the molecular weight of the MBP-ATG-SCO3550 is 130123.7 Da, the mass of the protein used for the assay is 1000 ng ($1000 * 10^{-9}$ g), total reaction volume is 200 μL ($200 * 10^{-6}$ L), and therefore the turnover number expressed in terms of dA/dt using equation (8) is as follows

Turnover number = $200 * 10^{-6} * 130123.734 / 6300 / 1000 * 10^{-9}$

Turnover number = 4130.9dA/dt

From Table 6.1, the change in absorbance per unit time for the first sample of ATP without poly U is 0.189 milliabsorbance per min. This implies,

Turnover number = $4130.9 * 0.189 * 10^{-3}$

= 0.8/min

Table 6.2. NTP requirements for MBP-ATGSCO3550 RNA unwinding activity.

NTP	Quantified signal values		
	Total	ssRNA	% of dsRNA unwound
AMP-PNP	11850	2762	23
ATP	8464	6022	71
CTP	9074	6691	74
GTP	9845	8547	87
UTP	10259	8327	81
dATP	9024	7071	78
dCTP	10159	7579	75
dGTP	10769	9369	87
TTP	8382	7308	87

Sample calculation of the % dsRNA unwound

% dsRNA unwound = quantified signal of ssRNA / total signal * 100

Therefore, using the signal values obtained for AMP-PNP

% of dsRNA unwound = 2762/11850 * 100

= 23 %

6.1. Calculation of the concentration of MBP-ATGSCO3550

Mass of the highest amount of protein used = 1000 ng

Molecular weight of the protein = 130123 Da

Moles of protein = mass / molecular weight

Moles of protein = $1000 * 10^{-9} / 130123$

= 7.7 ρmol

Total assay volume = 20 μL

Concentration of protein = moles / volume

This implies concentration = 7.7 ρmol / 20 μL

= 0.4 ρmol/μL

= 0.4 μM

6.2. Calculation of the moles of the dsRNA substrate (Fig. 3.6A) used

The molecular weight of the duplex RNA substrate in Daltons is given as

341 * Ls + 682 * Ld (modified from Frank 1997)

Where Ls is the length of ss RNA overhangs and Ld is the length of the duplex region.

The total length of the overhangs is 46 nucleotides and the length of the duplex region is 26.

Therefore, the weight of the substrate = 341* 81 + 682 * 14

= 37169 Da

Assuming that ss RNA and ds RNA absorb UV light to the same level,

1 A_{260} unit for the duplex RNA substrate = 40 μg /mL (1)

Average A_{260} for the substrate diluted 1:5 is 0.05.

Therefore, average A_{260} for the undiluted RNA substrate is 0.25 units (2)

From (1) and (2) above, the concentration of the RNA substrate is 10 μg / mL

$= 10 * 10^{-3}$ g/L

Moles of the RNA substrate used is given as

$$\frac{\text{concentration of the substrate in g/L * total volume of RNAsubstrate in L}}{\text{Molecular weight of the RNA substrate in g}}$$

Total volume of the RNA substrate used for the assay is 2 μL = $2* 10^{-6}$ L

Therefore using equation (2), moles of the RNA substrate = $\frac{10 * 10^{-3} * 2 * 10^{-6}}{37169}$

$= 0.5* 10^{-12}$ mol

= 0.5 ρmol

6.3. Molar proportion of MBP-ATGSCO3550 to the RNA substrate

Moles of the RNA substrate is 0.5 ρmol

Moles of the protein is 7.7 ρmol

Molar ratio of protein : substrate = 15.4 : 1

Therefore, the substrate is present at substoichiometric amounts in the assay.